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METHODS AND MEANS FOR PRODUCING PROTEINS WITH PREDETERMINED POST-TRANSLATIONAL MODIFICATIONS

Abstract:

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The present application provides proteins and methods for producing the same, wherein the proteins contain specific post-translation modifications. In particular, the application provides human etythropoietin containing a lower amount of sialic acid residues than epoetin alfa. Data supplied from the esp@cenet database - Worldwide

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(71) Applicant (for all designated States except US): CRU-CELL HOLLAND B.V. [NL/NL]; Archimedesweg 4, NL-2333 CN Leiden (NL).

(72) Inventors; and
(75) Inventors/Applicants (for US only): OPSTELTEN, Dirk, Jan, Elbertus [NL/NL]; Merelstraat 121, NL-2352 VC Leiderdorp (NL). KAPTEYN, Johan, Christiaan [NL/NL]; Akeleistraat 32, NL-6707 BZ Wageningen (NL). PASSIER, Petrus, Christianus, Johannes, Josephus [NL/NL]; Beekforel 5, NL-2318 MA Leiden (NL). BRUS, Ronald, Hendrik, Peter [NL/NL]; Baron Schimmelpenninck van de Oyelaan 8, NL-2252 EB Voorschoten (NL). BOUT, Abraham [NL/NL]; Van Swaanswijckstraat 38, NL-2751 XL Moerkapelle (NL).

(74) Agent: KLEIN, Bart.; Crucell Holland B.V, Intellectual Property Department, Postbus 2048, NL-2301 CA Leiden (NL).

(54) Title: METHODS AND MEANS FOR PRODUCING PROTEINS WITH PREDETERMINED POST-TRANSLATIONAL MODIFICATIONS

(57) Abstract: The present application provides proteins and methods for producing the same, wherein the proteins contain specific post-translation modifications. In particular, the application provides human etythropoietin containing a lower amount of sialic acid residues than epoetin alfa.

Title: Methods and means for producing proteins with
predetermined post-translational modifications.

FIELD OF THE INVENTION

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     The invention relates to the field of medicine. The
invention further relates to the production of proteins.
More particularly the present invention relates to the
production of recombinant proteins for use as a
10   therapeutically active constituent of a pharmaceutical
preparation.

BACKGROUND OF THE INVENTION

15        Recombinant expression systems for the production of
proteins are widely known. In general, human recombinant
proteins are manufactured with the use of a cellular
expression system. These systems range from bacteria, yeast
and fungi to plant cells, and from insect cells to
20   mammalian cells. Most of these cell-based systems are only
suited for a specific class of proteins. Consideration for
the production host and expression system of choice
generally relate to ease of use, cost of culturing, growth
characteristics, production levels and the ability to grow
25   on serum-free medium. Bacterial systems as well as yeast
systems are in many aspects the system of choice, since the
cost of culturing is low and they are in general easy to
use in comparison to other, higher eukaryotic systems.
Nevertheless, it is generally known that prokaryotic- and
30   lower eukaryotic systems differ in a number of aspects from
higher eukaryotic systems (such as mammalian cells). For
instance, certain post-translational modifications like

glycosylation do no occur in bacterial systems, while many
mammalian proteins depend on these post-translational
modifications to be therapeutically active. The cellular
expression systems mentioned above might also differ in the
5   capacity to exert other co- and post-translation
modifications such as folding, phosphorylation, γ-
carboxylation, and γ-hydroxylation. In general, the co- and
post-translational modifications are cell- and species
specific and depend on the expression system being applied.
10      Until now, Chinese Hamster Ovary (CHO) cells and Baby
Hamster Kidney (BHK) cells have been mainly used for the
production of recombinant human proteins and peptides for
use as a therapeutic active compound. CHO and BHK cells are
frequently the expression system of choice when a mammalian
15  expression system is required. These mammalian cells are
able to produce proteins efficiently and allow generally
for proper processing and folding. More recently, a human
cell line was proposed for production of human proteins.
The human post-translational modifications of human
20  proteins are thought to have a positive effect on the
pharmacokinetic and/or pharmacodynamic properties in
general (WO 00/63403).
        In the last number of years, studies have revealed
more about the complexities of differential post-
25  translational modifications of human proteins and the
potential implications on functions in the human body. For
example, relatively recent findings suggest that
differential glycosylation patterns of human proteins that
occur in the blood (so-called 'serum-type' modifications)
30  are different from the ones that occur in the cerebrospinal
fluid in the brain ('brain-type' modifications). This
difference may be the key issue that is of paramount

importance for the design of effective therapeutics. Taking
the difference in post-translational modifications found on
proteins that have a function in the serum as well as in
the brain as an example, it is now evident that new

5   expression systems are needed that take into account the
differential post-translational modifications on
therapeutic proteins that are required for a proper
activity in different parts of the human body as well as in
different types of disease. For example, proteins that

10  might have a beneficial effect on neural disorders should
have 'neural-like' post-translational modifications and
should be produced on expression systems having the ability
to produce proteins harboring such modifications. Proteins
that have these specific needs may be beneficial in the

15  treatment of all sorts of disorders, among which are the
diseases related to the Central Nervous System (CNS), the
peripheral nervous system and heart tissue.

        Disorders affecting the CNS encompass different kinds
of afflictions such as acute brain damage,

20  neurodegenerative diseases and other dysfunctions such as
epilepsy, schizophrenia and mood disorders. With the
increase of the number of elderly people in the worldwide
population in the next decades, an increasing number of
people become at risk of degenerative neurological diseases

25  related to the CNS. Two main examples of such disorders are
Parkinson's and Alzheimer's Disease. Other pathological
disorders that might afflict neural cells and tissues are
due to injuries that might be a result of hypoxia, seizure
disorders, neurotoxin poisoning, multiple sclerosis,

30  hypotension, cardiac arrest, radiation or hypoglycemia.
Neural injuries might also occur during surgical procedures
such as aneurysm repair or tumor resection. To date, the

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treatment of the CNS disorders has been mainly via administering pharmaceutical compounds. The main drawback that has been appreciated in the art is the limited ability to transport drugs across the blood-brain barrier and the

5   drug tolerance that is acquired by patients to whom these drugs are administered for prolonged periods of time. Other treatments for degenerative diseases related to the CNS include neurological tissue grafting: the use of fetal cells for neuro-transplantation has been explored. A clear

10  improvement in the condition of Parkinson's patients was reported by several groups after applying such techniques (Freed et al. 1992; Widner et al. 1992). Despite these successful reports, the use of large amounts of aborted fetal tissue for such purposes is hampered by significant

15  problems such as ethical and political issues. Moreover, fetal tissue is most likely never a homogeneous cell population, so it is not a well-defined source of cells, and the question remains whether there will be a sufficient and adequate constant supply of fetal tissue for these

20  purposes. Another method makes use of the fact that in the adult mammalian neural tissue multipotent neural stem cells exist, that are capable of producing progeny that differentiate into neurons and glia (Reynolds and Weiss 1992). Methods have been provided for the proliferation of

25  these stem cells to provide large numbers of neural cells that can differentiate into neurons and glia using various growth factors (U.S. Pat. No. 5,750,376; WO 94/10292). These findings have shown that there might be ways to treat neurological disorders such as degenerative afflictions by

30  the administration of stimulating factors such as recombinant growth factors and/or hormones.

Several investigators have found that nervous tissue
expresses high levels of one particular type of hormone,
known as Erythropoietin (EPO). Moreover, the EPO-receptor
(EPO-R) was also found to be expressed to high

5       concentrations in these tissues (Digicaylioglu et al. 1995;
Juul et al. 1997; Marti et al. 1997; Morishita et al.
1997). EPO, a protein famous for its role in
differentiating hematopoietic stem cells into red blood
cells, also seems to increase the number of neural progeny

10      that are generated from proliferated neural stem cells.
Several methods of inducing the differentiation of
multipotent neural cells into neurons and methods of
treating neurodegenerative diseases or acute brain injuries
by producing neurons from such cells using EPO are

15      described in U.S. Pat. No. 6,165,783. Thus, it has recently
become clear that EPO not only has a hematopoietic effect,
but that there also might be a role for EPO in neural
tissues. There is increasing evidence that non-erythroid
cells express the EPO-R and respond to recombinant EPO *in*

20      *vitro* and *in vivo*. For example, neural cell lines like NT2,
PC12 and SN6.10.2.2. express the EPO-R, and exposure of
PC12 cells to recombinant human EPO causes a rapid influx
of calcium from outside the cells and increases the
intracellular concentration of monoamines (Masuda et al.

25      1993). Recombinant human EPO was also found to augment
choline acetyltransferase (ChAT) activity in primary
cultured mouse septal neurons and in the cholinergic
hybridoma cell line, SN6.10.2.2. (Konishi et al. 1993). In
the developing and mature brain of rodents, monkeys and

30      humans expression of EPO and the EPO-R has also been
detected, (Marti et al. 1996; Dame et al. 2000; Yasuda et
al. 1993; Digicaylioglu et al. 1995; Morishita et al.

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1997). The expression of EPO and the EPO-R has been localized to neurons and glia cells in spinal cord and brain during fetal human development (Juul et al. 1998). The distribution of EPO and EPO-R proteins in fetal and

5    adult brain has been determined by immunohistochemistry (Juul et al. 1999a and 1999b). The synthesis of EPO in the brain seems to take place primarily in astrocytes (Marti et al. 1986; Masuda et al. 1994) and in neurons (Bernaudin et al. 2000).

10       Although the emphasis over the last years has been on the role of EPO in erythropoiesis, it was already reported by in 1978 (Peschle et al. 1978) that the production of EPO is not restricted to the fetal liver and adult kidneys, but also takes place inside the developing and adult brain. In

15   the human fetus EPO is expressed at a level that is comparable to that in the liver and kidneys, which are the most relevant sites for circulating EPO at this stage of development (Dame et al. 2001). Also in mice, it has been shown that there is a high constitutive level of EPO mRNA

20   in the brain compared to the kidneys and the liver (Marti et al. 1996). These observations suggested a role of EPO in the development of the CNS (Dame et al. 2001).

        EPO protein has also been detected in the cerebrospinal fluid (CSF) of human neonates and adults

25   (Juul et al. 1997; Buemi et al. 2000). The concentration of EPO in CSF is relatively high in neonates and decreases to lower but nevertheless detectable levels in adults (Juul et al. 1997; Marti et al. 1997). Compared to healthy individuals, patients with old cerebrovascular disease and

30   patients with depression have an elevated level of EPO in the CSF (Nakamura et al. 1998). That EPO is produced locally in the brain is strengthened by the observation

that EPO does normally not cross the intact blood-brain
barrier because there is no correlation between the
concentration of EPO in the serum and in the CSF (Marti et
al. 1997). This was further confirmed by the fact that the
5    intravenous administration of a total dose of 6,000 U of
recombinant human EPO in a human patient did not lead to an
increased concentration of EPO in the CSF (Buemi et al.
2000). Yet, studies in mice and rats have shown that EPO is
able to cross the blood-brain barrier when recombinant
10   human EPO is applied systemically (intra-peritoneal) at a
relatively very high dose of 5,000 U/kg body weight (Brines
et al. 2000; WO 00/61164). EPO also seems to be transported
into the brain in case of blood-brain barrier dysfunction,
e.g., in case of a traumatic brain injury. In that case, a
15   correlation between the levels of EPO in serum and CSF is
found (Marti et al. 1997).

        Interestingly, the expression of the EPO gene in the
kidneys as well as in the brain is increased under hypoxic
conditions. The expression is regulated by the
20   transcription factor hypoxia-inducible factor-1, which is
activated by a variety of stress signals, including
hypoxia. It has recently been demonstrated in mice that the
EPO mRNA levels markedly increase within 4 hours upon the
exposure to hypoxia (Chikuma et al. 2000). Yet, the EPO
25   mRNA levels in the kidneys decreased within 8 hours during
continuous hypoxia whereas the EPO mRNA levels in the
cerebrum remained the same. Hence, the regulation of EPO
expression is tissue-specific, which further strengthens
the hypothesis that EPO has tissue-specific functions that
30   are different between the brain and the bone marrow (Masuda
et al. 1999; Chikuma et al. 2000; Sasaki et al. 2001), and
that therefore EPO, besides its heamatopoietic function,

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also has other functions such as a neurotrophic role. The
expression of EPO in monkey- and mouse brain has been shown
to be 3 to 20-fold increased respectively, under hypoxic
conditions (Digicaylioglu et al. 1995; Marti et al. 1996).

5   Hypoxic effects on the expression of EPO have also been
demonstrated *in vitro*. In primary astrocyte cultures
hypoxia caused a more than 100-fold up-regulation of the
mRNA levels of EPO (Marti et al. 1996). Furthermore, it has
been found that the mRNA level of the EPO-R is also

10  upregulated under hypoxic conditions *in vivo*. This was
found in studies in which the middle cerebral artery of
rats was occluded and in which the increase of EPO-R mRNA
was detected by *in situ* hybridization in the periphery of a
cerebrocortical infarct (Sadamoto et al. 1998). It suggests

15  that neurons increase their sensitivity to EPO by
increasing their number of EPO-R under hypoxic and/or
ischemic conditions. Finally, it has recently been
confirmed by immunohistochemical methods that the
expression of immuno-reactive EPO and EPO-R is also

20  upregulated in fresh infarcts inside the human ischemic/
hypoxic brain (Sirén et al. 2001).

        Several studies have now demonstrated that EPO can act
as a neurotrophic factor. Neurotrophic factors are defined
as humoral molecules acting on neurons to influence their

25  development, differentiation, maintenance, and regeneration
(Konishi et al. 1993). Neurotrophic effects of EPO have
first been shown *in vitro*, in cultured neurons. For
example, it has demonstrated that recombinant human EPO, in
a dose-dependent manner, protects cultured embryonic rat

30  hippocampal and cerebral cortical neurons from glutamate
toxity (Morishita et al. 1997). Hypoxia-induced cell death
in cultures of postnatal rat hippocampal neurons also has

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been shown to be reduced by EPO (Lewczuk et al. 2000). In
addition, it has been shown that recombinant mouse EPO
protects cultured rat cortical neurons, but not astroglia
from glucose deprivation-induced hypoxia and from the
5   neurotoxic effects of (±)-α-amino-3-hydroxy-5-
methylisoxazole-4-propionic acid (Sinor and Greenberg
2000). Furthermore, recombinant human EPO has recently been
proven not only to protect neural cell cultures form
hypoxia but also from serum deprivation or kainic acid
10   exposure (Sirén et al. 2001). The neurothropic function of
EPO has also been shown in rats, which were treated with
soluble EPO-R that competed with the natural EPO-R for
binding endogenous EPO. These rats showed neural
degeneration and an impaired learning ability (Sakanaka et
15   al. 1998). The neurotrophic and neuroprotective effects of
EPO have also been studied in animal models with hypoxic-
ischemic injuries. For example, it has been shown that the
infusion of recombinant human EPO into the lateral
ventricles of gerbils prevented ischemia-induced learning
20   disability and rescued hippocampal CA1 neurons from lethal
ischemic damage in a dose-dependent manner (Sakanaka et al.
1998). In addition, it has been found that recombinant
human EPO, which was infused into the cerebroventricles of
stroke-prone spontaneously hypertensive rats with permanent
25   occlusion of the left middle cerebral artery, alleviated
the ischemia-induced place navigation disability and
supported neuron survival (Sadamoto et al. 1998). In a
similar model, it has been shown that systemic
administration (intra-peritoneal) of a high dose (5,000
30   U/kg body weight) of recombinant human EPO (Epoietin alpha,
Procrit, Ortho Biotech, Raritan, NJ) reduced the infarct
volume by ~75% at 24 h after occlusion of the middle-

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cerebral artery (Brines et al. 2000). In the same study, it was also observed that the treatment with EPO reduced immune damage in experimental autoimmune encephalomyelitis, as well as kainate-induced toxicity. Furthermore, the

5   effects of EPO have been studied in rabbits with subarachnoid hemorrhage-induced acute cerebral ischemia (Alafaci et al. 2000). It was found that recombinant human EPO that was systemically administered after the subarachnoid hemorrhage significantly reduced the number of

10  necrotic cortical neurons compared to the placebo controls.

In addition to the mentioned effects of EPO on erythropoiesis and neuroprotection, other roles of EPO have been described. The expression of EPO and its receptor are identified in endothelial cell *in vitro* and *in vivo*

15  (Anagnostou et al. 1994). *In vitro* experiments demonstrated that recombinant human EPO stimulates cell migration and proliferation in endothelial cell cultures. These *in vitro* results were further extended by the effect of human recombinant EPO on the stimulation of new blood vessel

20  formation in the chick chorioallantoic membrane (Ribatti et al. 1999). In accordance in mice, EPO was found to play an important role in the 17-beta-estradiol dependent angiogenesis in the uterine endometrium (Yasuda et al. 1998). Proliferative effects of EPO have also been shown on

25  muscle cells. In the myoblast C2C12 cell-line EPO enhanced the proliferation and reduced the differentiation and fusion into myotubes of these cells *in vitro* (Ogilvie et al. 2000). Furthermore, the receptor of EPO could also be detected on primary satellite cells isolated from skeletal

30  muscle from mice and on C2C12 cells at the mRNA and at the protein level. In addition EPO has been shown to stimulate

DNA-synthesis and proto-oncogene expression in rat vascular
smooth muscle cells.

An increased proliferation following recombinant human
EPO administration was also found on primary cultures of
5     neonatal rat cardiac myocytes (Wald et al. 1996). This
mitogenic effect of EPO was associated with a stimulatory
effect on $Na^+$-$K^+$-ATPase activity and was shown to be
secondary to the activation of tyrosine kinase and PKC.
These enzymatic pathways have also been described for other
10    cytokines in different tissues. A role for EPO on the
proliferation of cardiomyocytes was supported by knockout
studies. During embryogenesis EPO is expressed in the heart
and mice lacking EPO or EPO-R expression display cardiac
defects, demonstrated by ventricular hypoplasia and a
15    reduction in the number of proliferating cardiomyocytes and
increased apoptosis (Wu et al. 1999). Yu et al. (2001)
demonstrated that the cardiac phenotype in the EPO-R
knockouts could be rescued by crossing these mice with
transgenic mice, harboring the human EPO receptor gene. In
20    addition to the restoration of erythropoiesis, the cardiac
defect was corrected and apoptosis was markedly reduced.
Furthermore, also apoptosis in liver and brain was
significantly reduced in these mice.

Recent clinical studies demonstrated the beneficial
25    effects of EPO in patients with congestive heart failure
(CHF). CHF is defined as a heart disease, in which the
heart is not able to pump blood at a rate required by the
metabolizing tissues, or when the heart can do so only with
an elevated filling pressure. A high percentage of CHF
30    patients are anemic (low hemaglobin percentage) and a
correlation exists between the severity of the condition of
CHF and the degree of anemia. When patients with anemia in

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CHF were treated with EPO, a significant improvement with respect to cardiac function, renal function and a marked decrease was observed in the need for diuretics and hospitalization (Silverberg et al. 2000 and 2001). Although

5   the beneficial effects of EPO have been ascribed to its effect on erythropoiesis, direct cardiac effects, such as anti-apoptosis and angiogenesis may partially explain the positive outcome in these patients.

During ischemia/reperfusion injury, decreased cellular

10  oxidative phosphorylation leads to the failure of energy-rich phosphates, followed by an altered membrane potential, production of reactive oxygen species, cytokines and repression of protective gene products such as Nitric Oxide Synthase. In this process several adhesion molecules are

15  expressed on the endothelial cells in the affected area. These promote leukocyte adhesion to the endothelium followed by transmigration. Transmigration and subsequently activation of leukocytes leads to the production of toxic reactive oxygen species, proteases, elastases and is

20  generally followed by apoptosis, or other types of cell death (Carden and Granger 2000; Butcher 1991; Panes et al. 1999).

A certain family of glycoproteins, named selectins, play an important role in the initial steps of adhesion of

25  leukocytes to the endothelium in ischemia/reperfusion injury. There are three members in the selectin family: P-selectin, E-selectin and L-selectin. L-selectin is constitutively expressed on leukocytes, whereas P-selectin and E-selectin are found on the membrane upon activation.

30  P-selectin is present in Weibel-Palade bodies of endothelial cells and alpha-granules of platelets. Following ischemia/reperfusion or activation by different

agents, P-selectin is translocated to the cell surface,

within 10-20 minutes (Lorant et al. 1991; Weyrich et al.

1995), whereas E-selectin is expressed on endothelial cells

after *de novo* synthesis, which takes approximately 4-6

5   hours (Bevilacqua et al. 1989). Selectins initiate the

rolling of the leukocytes along the endothelium. P-selectin

is the most important selectin for this first step of

leukocyte rolling (Lefer and Lefer 1996). The rolling of

the leukocytes reduces the velocity of the leukocytes in

10  the bloodstream and allows a more firm interaction between

the leukocytes and the endothelium by other adhesion

molecules (integrins). Firm adhesion is followed by

transendothelial migration. Infiltration of the neutrophils

can be observed 3 hours following reperfusion. After this

15  time period, reperfusion injury with its resulting cell

death takes place (Armstead et al. 1997). Several ligands

of selectins have been identified. P-selectin glycoprotein

ligand-1 (PSGL-1) is a high affinty ligand for P-selectin

and to a lesser extent for L-selectin and E-selectin (Moore

20  et al. 1994). The oligosaccharides in PSGL-1 are recognized

by the lectin domain of the selectins.

It has been well established in the art that the

function of (recombinant) EPO depends heavily on the

glycosylation pattern of the oligosaccharides present on

25  the protein. The N-linked oligosaccharides of human EPO are

highly important for its well-known biological activity:

the stimulation of erythropoiesis (Takeuchi and Kobata

1991; Wasley et al. 1991). For instance, the sialic acids

protect the protein from being cleared through the hepatic

30  asialoglycoprotein receptor (Tsuda et al. 1990; Morimoto et

al. 1996). Furthermore, the highly branched N-linked                .

oligosaccharides are thought to increase the volume of the

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protein such that it is not easily filtered by the kidney

(Takeuchi et al. 1989; Misaizu et al. 1995). The multi-

antennary sugar structures may also have a role in

targeting of EPO to the bone marrow where erythropoiesis

5    occurs (Takeuchi et al. 1989). The role of the O-linked

oligosaccharides present on EPO is relatively unclear and

some data have suggested that there is only a limited role

for the O-linked sugar in EPO present in circulation and

that has an effect on hematopoiesis.

10       In general, human neural glycoproteins are

characterized by their glycosylation, which has been

referred to in literature as 'brain-type' glycosylation

(Margolis and Margolis 1989; Hoffmann et al. 1994). In

contrast to 'serum-type' glycosylated proteins (i.e.,

15   glycoproteins circulating in the blood) brain-type

glycosylated proteins characteristically possess complex-

type N-linked sugars that are modified with α1,3-linked

fucose attached to N-acetyl-glucosamine in lactosamine-type

antennae thereby forming Lewis x or sialyl-Lewis x

20   structures (Fig. 5). There are two types of Lewis x

structures: One with a terminal galactose residue and one

with a terminal N-acetyl-galactosamine (GalNac) residue. If

these terminal groups are linked to a sialic acid, the

Lewis x structure is called a sialyl Lewis x structure.

25   Another difference between serum-type and brain-type

oligosaccharides is that the latter often contain terminal

N-acetyl-glucosamine and/or terminal galactose, and may

include a terminal N-acetyl-galactosamine modification,

whereas serum-type oligosaccharides usually contain only

30   low amounts of such structures. It has also been suggested

that brain-type N-oligosaccharides characteristically

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contain high amounts of bisecting N-acetyl-glucosamine
(Hoffmann et al. 1994).

It is thus far unknown what the O-glycan structure is
in brain-type EPO, but the limited role for this
5    oligosaccharide in serum might imply that there is a more
important role for this type of glycosylation in brain-type
EPO. It is very likely as well, that in accordance with the
N-glycans, also the O-glycan has a differential
glycosylation pattern between serum-type and brain-type.
10   Oligosaccharides that are generally found on proteins
circulating in the serum contain often heavily
galactosylated structures. This means that a galactose is
linked to a peripheral N-acetyl-glucosamine thereby forming
a lactosamine structure. The glycoprotein is in this way
15   protected from endocytosis by the N-acetyl-glucosamine
receptors (i.e., receptors that recognize terminal N-
acetyl-glucosamine) present in hepatic reticuloendothelial
cells and macrophages (Anchord et al. 1978; Stahl et al.
1978). Serum-type oligosaccharides usually also contain
20   terminal sialic acids (also often referred to as neuraminic
acid) which protect the glycoprotein from clearance through
the asialoglycoprotein receptor. These clearance mechanisms
specifically apply to glycoproteins circulating in the
blood and are probably lacking in the human central nervous
25   system (CNS) (Hoffmann et al. 1994). A difference in
glycosylation is exemplified by transferrin that occurs in
significant amounts as asialotransferrin in the CSF but not
in that form in serum (Van Eijk et al. 1983; Hoffmann et
al. 1995). Transferrin is a protein capable of interacting
30   with iron via two iron-binding sites in the protein. Iron
uptake by cells occurs through receptor-mediated
endocytosis of the transferrin protein that is bound to

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iron. Human serum-type transferrin carries two N-
glycosylation sites (Asn413 and Asn611) that are generally
occupied by disialylated, bi-antennary oligosaccharide
chains. Most transferrin proteins seem to have 4 sialic

5   acid residues (i.e. two on each of the bi-antennary
chains). Serum-type transferrin does not contain
polylactosamines or fucose residues. A minor amount of
serum-type transferrin carries sialylated tri-antennary
oligosaccharides. It has been found that rarely also tetra-

10  antennary structures occur.

The glycosylation of transferrin is used as a marker
for carbohydrate-deficient glycoprotein syndromes (CDGS).
As a result there is extensive information in the
literature regarding glycosylation status. This includes

15  information of transferrin isolated from various human
sources including serum, cerebrospinal fluid (CSF),
amniotic fluid and synovial fluid. Apparently
differentially glycosylated forms are present in separate
parts of the human body strongly suggesting a differential

20  role of the different glycosylation patterns present on
transferrin in these separate tissues, as has been
discussed here for EPO.

In the case of EPO, one can also refer to a serum-type
EPO (or a 'renal-type', or a 'urinary-type' EPO) for the

25  protein that is produced in the kidney and that circulates
in the blood, as compared to EPO that is been produced by
other tissues such as the brain (brain-type).

EPO protein that was produced by cultured rat brain
cells was found to be significantly smaller than the EPO

30  protein present in circulation. This mass difference might
be the reason for the different biological roles in the
brain and in circulation. It was found, that although

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brain-type EPO produced on these cultured rat brains is
approximately 15% smaller than serum-type EPO (presumably
mainly due to differences in sialylation), this brain-type
EPO is more active *in vitro* in erythroid colony stimulation

5   at low ligand concentrations (Masuda et al. 1994). The
reason for this finding might be that, although the
affinity for brain-type EPO is higher towards the EPO-R,
the brain-type EPO will in its specific glycosylation form
never circulate long enough *in vivo* to exert the effects

10  that are normally brought about by the serum-type EPO
because this type of EPO is better protected against kidney
filtering and binding to the hepatic asialoglycoprotein
receptors, as discussed above. It is also generally known
by persons skilled in the art, that a low sialylation of

15  the EPO protein is beneficial for its affinity towards the
EPO-R. However, for circulating EPO involved in
erythropoiesis it is better to be protected against
clearance by heavy sialylation, than have a very high
affinity for the receptor.

20      There is a possible role for the sialyl Lewis x
modifications in oligosaccharides in binding to selectins
(Foxall et al. 1992). Sialyl Lewis x structures are
expressed on leukocytes and are rapidly expressed on
vascular endothelial cells and cardiac myocytes following

25  myocardial ischemia/reperfusion injury *in vivo* (Yamazaki et
al. 1993). Furthermore, sialyl Lewis x structures are also
induced on the surface of endothelial cells and
cardiomyocytes by hypoxia/reoxygenation *in vitro* (Seko et
al. 1996). Several studies in the art have indicated the

30  importance of selectins and sialyl Lewis x structures for
the adhesion of leukocytes in models of ischemia/
reperfusion. The sialyl Lewis x oligosaccharide Sle$^x$-OS was

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shown to be cardioprotective in a feline model of ischemia/reperfusion by reducing cardiac necrosis by 83% (Buerke et al. 1993). In addition reduced adhesion to the endothelium was observed in this model using Sle$^x$-OS.

5   Furthermore, in a similar ischemia/reperfusion model, treatment with Sle$^x$-OS also resulted in a 100% recovery in cardiac function, compared to 71% recovery of cardiac function with saline. In dogs subjected to myocardial infarction/reperfusion, treatment with Sle$^x$-OS demonstrated

10  cardioprotection by a 55% reduction in infarct size (Flynn et al. 1996).

For many years a recombinant version of the serum-type EPO has been used in patients suffering from kidney failure, anemia and/or in patients undergoing heavy surgery

15  resulting in dramatic blood-loss. Generally, this recombinant EPO results in an increased production of red blood cells from hematopoietic stem cells in the bone marrow. It is well established in the art that the recombinant EPO had to fulfill all requirements of a stable

20  protein that could circulate in the bloodstream for a sufficient amount of time to enable the induction of erythropoiesis. The system of choice thus far has been a production platform on CHO cells from which the higher sialylated EPO forms were purified and used to prepare a

25  medicament for the treatment of patients that suffer from the disorders resulting from a low-red blood cell level. Other cells that were used for these purposes were BHK cells. Apparently CHO and BHK cells are capable of generating the correct glycosylation (sialylation) patterns

30  on the recombinant product to yield positive effects in human renal-failure patients. However, CHO produced EPO does not have the characteristic features of an EPO

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molecule that is active in the brain or in tissues that
involve selectin-based transport. Therefore, urinary- or
serum-type EPO (produced on cells such as CHO or BHK) is
relatively useless in the treatment of disorders related to

5    the Central- or Peripheral Nervous system as well as in the
treatment of afflictions related to ischemia/reperfusion
induced disorders. This, because of its glycosylation
pattern that is not suited for these kind of tissues, and
also because it renders side effects such as an increase in

10    the number of red blood cells (erythropoiesis) due to its
strong hematopoietic activity.

No proper production platforms are present in the art
that are able to produce significant amounts of recombinant
proteins harboring tissue-specific predetermined post-

15    translational modifications such as a brain-type
glycosylation on recombinant EPO, and that can be used in
the manufacturing of medicaments for the treatment of
patients suffering from disorders that require such
proteins, as well as the treatment of patients at risk of

20    developing such disorders.

BRIEF DESCRIPTION OF TABLES AND FIGURES

Table I. Overview of the marker proteins that can be used to characterize cells.

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Table II. Positive control tissues that can be used for some of the marker proteins depicted in Table I.

Table III. Detailed information (Supplier and Catalogue
10    numbers) of antibodies directed to marker proteins that were used to characterize the PER.C6™ cell line.

Table IV. Score of the presence of the marker proteins on PER.C6.

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Table V. Monosaccharide composition of the N-linked sugars of PER.C6-EPO and Eprex.

Table VI. Assignments of MS peaks observed for the
20    molecular ions of desialylated N-glycans released by N-glycanase F from EPO produced in DMEM by EPO producing PER.C6 clone P7. Peaks with mass (m/z) values that are also found in Eprex are underlined and indicated in bold.

25    Table VII. Assignments of MS peaks observed for the molecular ions of desialylated N-glycans released by N-glycanase F from EPO produced in DMEM by EPO producing PER.C6 clone P8. Peaks with mass (m/z) values that are also found in Eprex are underlined and indicated in bold.

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Table VIII. FUT activities in CHO and PER.C6 cells.

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Figure 1. Mass spectra of the N-linked sugars of Eprex, P7-EPO (pools A, B, and C), and P8-EPO (pools A, B, and C). (A) Eprex; (B) P7, pool A; (C) P7, pool B; (D) P7, pool C; (E) P8, pool A; (F) P8, pool B; and (G) P8, pool C.

Figure 2. Sialic acid content of PER.C6-EPO and CHO-EPO.

Figure 3. Lewis x glycan structures present on PER.C6-EPO.

Figure 4. Lewis x structures expression at the PER.C6 cell surface.

Figure 5. Schematic representation of Lewis x and Sialyl Lewis x structures.

Figure 6. Effect of PER.C6-EPO and Eprex on erythropoiesis *in vivo*.

Figure 7. Infarct volumes in untreated rats (control) and Eprex and PER.C6-EPO treated rats based on the ADC maps (Fig. 7A) and the T2 maps (Fig. 7B) generated at 24 h after the onset of reperfusion, using MRI.

Figure 8. Concentration of Eprex at the indicated time-points after a single i.v. injection of 150 eU of Eprex in three animals.

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SUMMARY OF THE INVENTION

The present invention provides methods for identifying, selecting and obtaining mammalian cells that
5    are capable of producing proteinaceous molecules, such as peptides and proteins comprising post-translational modifications, wherein said post-translational modifications are predetermined and brought about by the mammalian cell in which the proteinaceous molecule is
10   expressed. The invention further provides methods for obtaining and producing proteinaceous molecules, such as erythropoietin (EPO), using mammalian cells obtainable according to methods of the present invention and on mammalian cells that have been obtained on the basis of
15   their ability to produce proteins and/or post-translational modifications that are elusive for the predetermined post-translational modification that is desired.

In one preferred embodiment, the present invention provides mammalian cells that have neural characteristics
20   and properties such that significant amounts of recombinant proteins can be produced that harbor 'neural- or brain-type' properties. The production of recombinant proteins, like brain-type EPO, carrying specific predetermined post-translational modifications, is now feasible by using the
25   methods and means of the present invention.

The present invention furthermore provides methods for purifying proteinaceous molecules, wherein said proteinaceous molecules are purified from cell culture on the basis of the predetermined post-translational
30   modification present on the molecule, said predetermined post-translational modification being brought about by the mammalian cell on which the molecule was produced.

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The present invention furthermore provides for use of a composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin,

5    or a collection of one or more fractions of erythropoietin molecules sialylated to a varying degree, for the preparation of a medicament for the treatment of a disorder selected from the group consisting of ischemia, a reperfusion injury, a hypoxia-induced disorder, an

10   inflammatory disease, a neurodegenerative disorder, and acute damage to the central- or peripheral nervous system, wherein said composition of erythropoietin-like molecules has on a protein content basis a lower erythropoietic activity *in vivo* than epoetin alfa. The present invention

15   also provides pharmeceutical compositions comprising such erythropoietin-like molecules. The invention also provides methods for treatment or prevention said disorders, comprising administering said compositions.


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DETAILED DESCRIPTION


The present invention provides a method for identifying a mammalian cell capable of producing a

25   proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: a) analyzing the post-translational modification on a protein produced by said mammalian cell; and b) determining whether said protein comprises said

30   predetermined post-translational modification.

In another embodiment the invention provides a method for selecting a mammalian cell capable of producing a

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proteinaceous molecule comprising a predetermined post-
translational modification, said method comprising the
steps of: a) analyzing the presence or absence of a tissue
specific marker or a combination of tissue specific markers
5    in said mammalian cell or on the cell surface of said
mammalian cell, which marker or combination of said markers
is indicative for said predetermined post-translational
modification to be present on said proteinaceous molecule;
and b) selecting said mammalian cell on the basis of the
10   presence or absence of said tissue specific markers.

In yet another embodiment, the invention provides a
method for obtaining a mammalian cell from a heterogeneous
cell population, said mammalian cell being capable of
producing a proteinaceous molecule comprising a
15   predetermined post-translational modification, said method
comprising the steps of: a) sorting cells on the basis of
the post-translational modifications on proteins produced
by said cells in said heterogeneous cell population; and b)
selecting the cells capable of producing proteins
20   comprising said predetermined post-translational
modification.

In another embodiment, the invention provides a method
for identifying a mammalian cell capable of producing a
proteinaceous molecule comprising a predetermined post-
25   translational modification, said method comprising the
steps of: providing said mammalian cell with a nucleic acid
encoding a protein capable of comprising post-translational
modifications, in such a way that said mammalian cell
harbors said nucleic acid in an expressible form; culturing
30   said mammalian cell under conditions conducive to the
production of said protein; analyzing the post-
translational modification on said protein produced by said

mammalian cell; and determining whether said post-
translational modification present on said protein
comprises said predetermined post-translational
modification. Whereas in yet another embodiment, the
5   invention provides a method for identifying a mammalian
cell capable of producing a proteinaceous molecule
comprising a predetermined post-translational modification,
said method comprising the steps of: providing said
mammalian cell with a nucleic acid encoding said
10  proteinaceous molecule capable of comprising post-
translational modifications, in such a way that said
mammalian cell harbors said nucleic acid in an expressible
form; culturing said mammalian cell under conditions
conducive to the production of said proteinaceous molecule;
15  analyzing the post-translational modification on said
proteinaceous molecule produced by said mammalian cell; and
determining whether said post-translational modification
present on said proteinaceous molecule comprises said
predetermined post-translational modification.

20      A proteinaceous molecule as used herein refers to, but
is not limited to, molecules such as peptides, polypeptides
and proteins, as well as to mutants of peptides,
polypeptides and proteins (molecules comprising deletions,
pointmutations, swaps and/or chemically induced
25  alterations). It also refers to peptides, polypeptides and
proteins carrying tags and/or other proteinaceous and non-
proteinaceous labels (e.g., radio-active compounds). An
example of such a protein is human EPO, which has besides
the renal- or serum-type form, other phenotypes such as a
30  brain-type form. Other, non-limiting examples of classes of
proteins that have certain characteristics that possibly
play an important role in the functionality of the protein

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in certain tissues and that should (when recombinantly
expressed) harbor the predetermined post-translational
modifications for a proper function are monoclonal
antibodies, neurotrophins, cytokines, insulin-like growth
5    factors, TGF-β like growth factors, fibroblast growth
factors, epidermal growth factors, heparin binding growth
factors, tyrsosine kinase receptor ligands and other
trophic factors. Most of these factors are associated with
disease syndromes, and therefore most of the proteins might
10   be used in recombinant form in the treatment of humans,
provided that the proteins harbor the post-translational
modifications necessary to be active *in vivo*. These
proteins should therefore be produced on expression systems
that are capable of providing the required post-
15   translational modifications. Examples of such proteins are,
but are not limited to, transferrin, Nerve Growth Factor
(NGF), Brain-derived neurotrophic factor, Neurotrophin-3, -
4/5 and -6, Ciliary neurotrophic factor, Leukemia
inhibitory factor, Cardiotrophin-1, Oncostatin-M, several
20   Interleukins, GM-CSF, G-CSF, IGF-1 and -2, TGF-β, Glial-
derived neurotrophic factor, Neurturin, Persephin,
Myostatin, Fibroblast Growth Factor-1, -2 and -5,
Amphiregulin, Acetylcholine receptor inducing activity,
Netrin-1 and -2, Neuregulin-2 and -3, Pleiotrophin,
25   Midkine, Stem Cell Factor (SCF), Agrin, CSF-1, PDGF and
Saposin C. Monoclonal antibodies refer to human and
humanized antibodies, to parts thereof, and to equivalents
such as single chain Fv (scFv) fragments, Fab fragments,
CDR regions, variable regions, light chains and heavy
30   chains. Antibodies may be bispecific, trispecific, and so
forth; either in naked form or conjugated to antigenic

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moieties, toxins, fluorescent markers, radiolabels, and the
like.

A post-translational modification as used herein
refers to any modification that is present on or in said
proteinaceous molecule. It refers to modifications that are
introduced during or subsequent to the translation of said
molecule from RNA *in vivo* or *in vitro*. Such modifications
include, but are not limited to, glycosylation, folding,
phosphorylation, γ-carboxylation, γ-hydroxylation,
multimerization, sulphide bridging and for instance
processing events such as the clipping-off or the addition
of one or more amino acids. A predetermined post-
translational modification as used herein refers to any
post-translational modification that is useful for the
selected treatment. According to a preferred embodiment,
predetermined post-translational modification refers to a
form of modification that makes the modified protein
particularly useful to treat disorders of specific tissues,
organs, compartments and/or cells of a human or animal
body. Preferably, the proteinaceous molecule carrying such
predetermined post-translational modification is less
active in a tissue, organ, compartment and/or cell wherein
action is less desired. Even more preferred, the
proteinaceous molecule carrying such predetermined post-
translational modifications does not exert any significant
effect (such as detrimental- or other undesired side-
effects) other than the tissue, organ, compartment and/or
cell that is to be treated. According to one embodiment,
the predetermined post-translational modification causes
the protein comprising the predetermined post-translational
modification to be cleared from the blood more rapidly,
e.g., to reduce adverse side effects. The predetermined

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post-translational modification can be fully understood in detail in advance, but can also be generally referred to as being a desired state that is required for a proper and wanted activity of the proteinaceous molecule comprising
5    such predetermined post-translational modification, meaning that the detailed modifications present on the proteinaceous molecule of interest might not be fully understood and/or defined, but nevertheless hold activity features that are desired. The several glycosylation
10   modifications in O- and/or N-glycans that might be present on a proteinaceous molecule, that might, but do not have to be necessarily desired, are exemplified by structures such as Lewis x, sialyl Lewis x, GalNac, GlcNac, LacdiNAc, $\alpha$1,3-linked fucose attached to N-acetyl-glucosamine, terminal N-
15   acetyl-glucosamine, terminal galactose, bisecting N-acetyl-glucosamine, sulphate group and sialic acid.

   The mammalian cells of the present invention are preferably human, for the production of human proteins to produce proteins that most likely carry mammalian-, and
20   preferably human, characteristics. To produce proteinaceous molecules that should have neural post-translational modifications, it is preferred to use cells that have neural characteristics, such as protein markers that are indicative for neural cells. This does not exclude that a
25   non-neural cell might be extremely useful in producing proteins comprising neural-type post-translational modifications. It depends on the protein activity that is required, to select, identify or obtain a cell that is capable of producing such post-translational modifications.
30   The presence of certain cell-type specific marker proteins in or on a cell does also not exclude the possibility that a certain type of cell is capable of producing a protein

carrying the desired post-translational modifications,
although this cell expresses some unrelated protein
markers. It depends on the selection criteria, as well as
on the method for selecting (determining the protein
5    markers and/or the post-translational modifications present
on proteins produced in the cell) whether a certain cell is
suitable for producing the proteinaceous molecule of
interest.

        Since it is required to produce large quantities of
10   proteins when these will be applied in therapeutic
settings, it is preferred that the mammalian cells of the
invention are immortalized. Immortalization can be brought
about in many ways. Examples of methods to obtain
immortalized cells are actively transforming a resting cell
15   into a dividing cell by the addition of nucleic acids
encoding transforming and/or immortalizing proteins, or
through chemical treatment through which endogenous
proteins might become transforming, or by taking cells from
tumor material. One preferred method to immortalize non-
20   tumorous cells is by the addition of the E1 region of
adenovirus as was shown for cell lines such as HEK293, 911
and PER.C6. Also the presence of certain Human
Papillomavirus (HPV) proteins might render a cell
immortalized (HeLa cells). The addition of certain viral
25   proteins, such as E1 from adenovirus might be beneficial
for the production of recombinant proteins, since many of
such proteins have transcription-activating features, as
well as anti-apoptotic effects. Moreover, they might add to
the post-translational modifications in and/or on the
30   proteinaceous molecules that are produced in the cells in
which these transforming/immortalizing proteins are
expressed. For instance, E1A of adenovirus might induce the

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expression of certain endogenous proteins that have a role in the addition of post-translational modifications on the recombinant proteins to be produced. Moreover, a direct effect in these processes can also not be excluded.

5    The present invention provides a method for producing a proteinaceous molecule comprising a predetermined post-translational modification, comprising the steps of: providing a mammalian cell obtainable by methods according to the invention, with a nucleic acid encoding said
10   proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form; and culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule.

In one embodiment of the invention, the invention
15   provides a method for producing a proteinaceous molecule comprising a predetermined post-translational modification, comprising the steps of: identifying a mammalian cell having the ability to provide the proteinaceous molecule with said predetermined post-translational modification;
20   providing said mammalian cell with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form; and culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule.

25    In another embodiment, the invention provides a method for producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: identifying a mammalian cell having the ability to provide said proteinaceous molecule
30   with said predetermined post-translational modification; providing said mammalian cell with a nucleic acid encoding said proteinaceous molecule in such a way that said

mammalian cell harbors said nucleic acid in an expressible form; culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule; analyzing said post-translational modifications on said

5      proteinaceous molecule so produced; and determining whether said post-translational modification present on said proteinaceous molecule comprises said predetermined post-translational modification. A suitable cell line for the methods for producing proteinaceous molecules according to

10     the invention is PER.C6, deposited under No. 96022940 at the European Collection of Animal Cell Cultures at the Center for Applied Microbiology and Research.

The invention moreover provides methods for producing a proteinaceous molecule comprising a predetermined post-

15     translational modification, said method comprising the steps of: providing a mammalian cell obtainable by a method according to the present invention, with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an

20     expressible form; culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule, and purifying said proteinaceous molecule from the mammalian cell culture.

In another embodiment, the present invention provides

25     methods for producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: providing a mammalian cell obtainable by a method according to the present invention, with a nucleic acid encoding said proteinaceous molecule in

30     such a way that said mammalian cell harbors said nucleic acid in an expressible form; culturing said mammalian cell under conditions conducive to the production of said

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proteinaceous molecule; analyzing said post-translational
modifications on said proteinaceous molecule so produced;
and determining whether said post-translational
modification present on said proteinaceous molecule
5    comprises said predetermined post-translational
modification.

       Preferably, said methods for producing proteinaceous
molecules comprise the extra step of purifying said
proteinaceous molecule from the mammalian cell culture.
10   More preferred are methods for producing a proteinaceous
molecule in a mammalian of the invention, wherein said
mammalian cell is immortalized, and wherein said
immortalization is brought about as discussed above.
Immortalization can take place prior to the identification
15   of the obtained mammalian cell, but might also take place
after the proper cell for proper post-translational
modifications is identified, selected and/or obtained.

       Purification as used herein might be performed by
using conventional methods that have been described in the
20   art, however, it is preferred to use purification methods
that comprise a step in which the post-translational
modifications present in and/or on said proteinaceous
molecules are employed. Even more preferred are
purification methods that comprise a step in which the
25   predetermined post-translational modifications present in
and/or on said proteinaceous molecules are employed. When
affinity purification methods are applied, it is preferred
to use antibodies or other binders, such as lectins
specific for particular carbohydrate moieties and that are
30   directed against certain types of post-translational
modifications. Examples of such antibodies are antibodies
directed against (sialyl) Lewis x structures, lacdiNac

structures or GalNac Lewis x structures. Using such
antibodies enables one to purify the (recombinant) proteins
such that a high percentage of the purified protein carries
the desired predetermined post-translational modification.

5      Even more preferred are methods in which the proteinaceous
molecule is purified to homogeneity. Examples of methods
for purification of proteins from mammalian cell culture
are provided by the present invention and encompass for
instance affinity chromatography methods for the

10     purification of brain-type glycosylated EPO by using
antibodies directed against Lewis x structures present in
the N-glycans of the recombinantly produced product.

       The present invention provides a pharmaceutically
acceptable composition comprising a proteinaceous molecule

15     having a predetermined post-translational modification,
obtainable according to methods of the present invention,
and a pharmaceutically acceptable carrier. Pharmaceutically
acceptable carriers as used herein are exemplified, but not
limited to, adjuvants, solid carriers, water, buffers, or

20     other carriers used in the art to hold therapeutic
components, or combinations thereof. In a preferred
embodiment said proteinaceous molecule in said
pharmaceutically acceptable composition is erythropoietin.
Even more preferred, said erythropoietin has a lower

25     erythropoietic effect as compared to erythropoietin not
having said predetermined post-translational modification.
According to the invention, erythropoietin produced in
cells with neural protein markers acquires a post-
translational modification that is active in neural tissue

30     or on neural cells. However, the post-translational
modifications are not comparable to the post-translational
modifications seen on EPO that circulates in the blood. The

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erythropoietic effects of the EPO produced on cells with
the neural protein markers is significantly lower, most
likely due to the absence of a high percentage of sialic
acids, and/or to the presence of brain-type features such
5    as Lewis x structures and terminal galactosides. This is
advantageous, since such a brain-type EPO can be used in
relatively high dosages in the treatment of disorders
related to neural tissue or in the treatment of tissue
damaged by ischemia (such as an ischemic heart), but that
10   does not has any significant effect on erythropoiesis. Such
disadvantageous effects are appreciated in the art
(Wiessner et al. 2001). The invention provides recombinant
erythropoietin comprising at least one post-translational
modification selected from the group consisting of: a
15   sialyl Lewis x structure, a Lewis x structure, a α1,3-
linked fucose attached to N-acetyl-glucosamine, a LacdiNAc
structure, a terminal N-acetyl-glucosamine group and a
terminal galactose group. Preferably, said recombinant
erythropoietin is produced on a mammalian cell obtainable
20   according to the present invention. Said recombinant
erythropoietin could be produced on PER.C6. The present
invention further provides the use of PER.C6 for the
production of a proteinaceous molecule comprising a
predetermined post-translational modification, wherein it
25   is preferred that said proteinaceous molecule is rapidly
cleared from the blood and/or used in high dosage. In the
case of EPO, produced on PER.C6, for example high dosage
may be used to treat or prevent acute damage associated
with hypoxia, while limiting the adverse side effects of
30   erythropoiesis.

In one embodiment of the present invention, the
proteinaceous molecules of the present invention are

suitable for the treatment of a human or a human body by
surgery, therapy or diagnosis. Preferably said
proteinaceous molecules such as EPO are used for the
manufacture of a medicament for the treatment of hypoxia-
5    induced disorders, neurodegenerative afflictions, or acute
damage to the central- or peripheral nervous system. In
another preferred embodiment, said proteinaceous molecules
such as EPO are used for the manufacture of a medicament
for the treatment of ischemia/reperfusion injuries. In yet
10   another preferred embodiment, said proteinaceous molecules
such as EPO are used for the manufacture of a medicament
for the treatment of immune disorder and/or inflammatory
disease.

     Methods and compositions are disclosed herein for the
15   production and manufacturing of recombinant proteins. The
invention is particularly useful for the production of
proteins that require co-translational and/or post-
translational modifications such as glycosylation and
proper folding and relates furthermore to the use of human
20   cells capable of producing brain-type co- and/or post-
translational modifications on proteinaceous molecules.
These cells can for instance be used for the production of
human glycoproteins with neural features that might be
therapeutically beneficial, due to their neural features.
25        The present invention describes the use of a human
cell line with neural characteristics that modifies
recombinantly expressed proteins with neural properties
such as 'brain-type' or 'neural-type' post-translational
modifications such as glycosylation, phosphorylation or
30   folding. An example of such a cell line, named PER.C6™
(U.S. Pat. No. 6,033,908), was generated by the
immortalization of human embryonic retina cells using a

construct harboring the Adenovirus type 5 E1 genes. PER.C6 cells have proven to be particularly suitable for the production of recombinant human proteins, since high yields of proteins such as the human EPO and fully human

5    monoclonal antibodies could be obtained (described in WO 00/63403). In general, the advantage of using PER.C6 for the production of recombinant proteins is the high level of production and the fact that the cells can be cultured either in an adherent fashion or in suspension in medium

10   without serum or serum-derived components to very high densities.

The present invention discloses that PER.C6 cells express neural marker proteins, by showing that PER.C6 cells can be stained with specific antibodies against

15   vimentin, synaptophysin, neurofilament, glial fibrillary acidic protein (GFAP), and neural cell adhesion molecules such as N-CAM and CD56. The presence of these marker proteins, as well as the morphological characteristics of the cells (e.g., an epithelial phenotype with large nuclei

20   and many slender cytoplasmic processes well known to persons skilled in the art of histology) indicate that PER.C6 cells are of neural origin.

The invention further discloses that recombinant proteins produced by PER.C6 cells can acquire certain

25   tissue specific features such as neural characteristics (e.g., post-translational modifications such as glycosylation). This is exemplified by the production of a protein that harbors so-called brain-type oligosaccharides. It is shown that human EPO produced by PER.C6 cells is

30   modified with N-linked sugars that significantly differ from the N-linked sugars found in human urinary EPO or in recombinant human EPO produced by Chinese Hamster Ovary

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(CHO) cells or Baby Hamster Kidney (BHK) cells. Human urinary EPO and recombinant human EPO produced in CHO and BHK cells contain glycosylation structures that can be referred to as 'renal-type' or 'serum-type' oligo-

5    saccharides. Typically, the N-linked sugars of these CHO- and BHK-EPO preparations are highly branched, highly galactosylated, and highly sialylated, whereas they lack peripheral α1,3-linked fucose (Tsuda et al. 1988; Takeuchi et al. 1988; Nimtz et al. 1993; Watson et al. 1994; Rahbek-

10   Nielsen et al. 1997).

Herein, the nature of the oligosaccharides linked to human EPO produced on PER.C6 has been elucidated and shows that the oligosaccharides of PER.C6-produced human EPO differ significantly from the oligosaccharides present in

15   human urinary EPO and recombinant human EPO produced in CHO and BHK cells. Firstly, the average sialic acid content of the oligosaccharides of PER.C6-produced human EPO is significantly lower than the average sialic acid content of human urinary EPO or recombinant human EPO (from CHO and

20   BHK). Brain-type EPO does not circulate in the blood and it might very well be the reason that this EPO form does not need heavy sialylation for its protection against clearance. The very low sialic acid content in PER.C6- produced human EPO is indicative of the presence of N-

25   linked oligosaccharides that contain terminating galactose and/or N-acetyl-galactosamine and/or N-acetyl-glucosamine. Secondly, N-acetyl-galactosamine is found in significant amounts in the N-linked sugars of PER.C6-produced human EPO, whereas N-acetyl-galactosamine is almost absent in the

30   N-linked sugars of human urinary EPO and recombinant human EPO produced by CHO cells. Only trace amounts of N-acetyl- galactosamine have been reported to occur in the N-linked

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sugars in a few batches of recombinant human EPO produced
in BHK cells (Nimtz et al. 1993). Third, the N-linked
sugars of human EPO produced in PER.C6 cells are found to
contain a very high amount of fucose. A fraction of the
5    fucoses is α1,3-linked to a peripheral N-acetyl-glucosamine
thereby forming a so-called Lewis x structure (Fig. 5).
Lewis x structures have never been reported to occur in
human urinary EPO or in recombinant human EPO produced in
CHO and BHK cells. Importantly, the (sialyl) Lewis x
10   structures present on EPO might as a consequence relate to
a role in EPO binding to selectins and a further role in
cardioprotection. Such structures might be beneficial and
perhaps indicative for a possible role for EPO in direct
cardiac effects as discuused above. Taken together, the N-
15   linked oligosaccharides present on PER.C6-produced human
EPO have the strong characteristics of brain-type
oligosaccharides.

    Because the protein-linked oligosaccharides have a
great impact on the physicochemical properties of the
20   polypeptide such as tertiary conformation, solubility,
viscosity, and charge, PER.C6-produced human EPO has
physicochemical properties that differ significantly from
human urinary EPO and recombinant human EPO produced by CHO
and BHK cells (Toyoda et al. 2000). Clearly, PER.C6-
25   produced human EPO is less charged than human urinary EPO
and recombinant human EPO produced by CHO and BHK cells due
to a lower sialic acid content and it is more hydrophobic
due to the very high fucose content. As a result, the
average pI of PER.C6-produced human EPO is significantly
30   higher than the average pI of human urinary EPO or
recombinant human EPO produced by CHO and BHK cells.
Because the glycans of EPO, in particular the sialic acids,

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also have an influence on the binding to the EPO receptor,
it is expected that PER.C6-produced human EPO has a
different affinity for the EPO receptor than human urinary
EPO and recombinant human EPO produced by CHO and BHK

5   cells.

The present invention furthermore discloses the use of
brain-type proteins produced in neural human cells for the
treatment of ischemia/reperfusion injury in mammals and
especially in humans. Ischemia/reperfusion injury as used

10  herein is defined as the cellular damage that occurs after
reperfusion of previously viable ischemic tissues.
Ischemia/reperfusion injury is associated with, for
example, but not limited to thrombolytic therapy, coronary
angioplasty, aortic cross clamping, cardiopulmonary bypass,

15  organ or tissue transplantation, trauma and shock.

The present invention provides the use of therapeutic
proteins, produced in mammalian cells, with brain-type
oligosaccharides. These brain-type oligosaccharides
comprise in particular Lewis x structures, sialyl Lewis x

20  structures, or derivatives thereof containing the (sialyl)
Lewis x structure, for the treatment of
ischemia/reperfusion injury in mammalian subjects such as
humans. The presence of (sialyl) Lewis x structures on
recombinant proteins targets these proteins to the injured

25  site of ischemia/reperfusion and thereby exerting their
ischemia/reperfusion protective effect more effectively
than proteins containing no (sialyl) Lewis x structures.
The presence of brain-type oligosaccharides on
recombinantly expressed proteins is exemplified in the

30  present invention by Erythropoietin (EPO), which is
produced on PER.C6 cells. This particular type of EPO
contains the Lewis x as well as the sialyl Lewis x

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structures. In the present invention experiments are
described that show the superiority of PER.C6 brain-type
(or neural-type) EPO compared to serum-type (or renal-type)
EPO with respect to the cardioprotective function in *in*
5   *vivo* models of cardiac ischemia/reperfusion injury.
        An advantage provided by the present invention is that
PER.C6-produced human EPO is less active in stimulating
erythropoiesis *in vivo* than serum-type human EPO such as
recombinant human EPO produced in CHO and BHK cells, which
10  is currently used as a therapeutic drug to treat anemia in
human beings. This means that when applied intravenously at
the same dosis (i.e., equal amount of protein per kg body
weight), PER.C6-produced human EPO causes a smaller
increase in red blood cell production than the highly
15  sialylated fraction of recombinant human EPO that is
produced in CHO or BHK cells. As a result thereof, PER.C6-
produced human EPO causes a smaller increase in the
hematocrit value *in vivo* than the highly sialylated
fraction of recombinant human EPO produced in CHO and BHK
20  cells, when applied intravenously at the same dose. The
poor effect of PER.C6-produced human EPO on erythropoiesis
is most likely due to a relatively short half-life of the
protein in the blood circulation, and/or due to an impaired
targeting signal that directs the protein to erythroid
25  progenitor cells in the bone marrow, and/or due to a low
affinity of the protein for the EPO-R on erythroid
progenitor cells. The impaired functioning of PER.C6-
produced human EPO to stimulate erythropoiesis is a direct
effect of its oligosaccharide composition, which is, as
30  described above, significantly different from the
oligosaccharide composition of serum-type EPO such as human

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urinary EPO and recombinant human EPO produced in CHO and
BHK cells.

   Another important advantage presented by the present
invention is that PER.C6-produced human EPO has a
5   neurotrophic activity. PER.C6-produced EPO gives the EPO
protein physicochemical and/or pharmacokinetic and/or
pharmacodynamic advantages in functioning as a neurotrophic
and/or neuro-protecting agent. PER.C6-produced EPO has
higher affinity for neural cells and for the EPO-R on
10   neural cells than the highly sialylated serum-type
glycosylated human recombinant EPO produced in CHO and BHK
cells. Recombinant human EPO produced on non-neural cells
(Goto et al. 1988) has a lower affinity for the EPO-R on
neural cells than for the EPO-R on erythroid progenitor
15   cells (Musada et al. 1993 and 1994).

   The neuroprotective role of EPO clearly opens new
possibilities for the use of recombinant human EPO as
neuroprotective therapy in response to toxic chemicals that
may be induced by inflammation or by hypoxia and/or
20   ischemia, or in neurodegenerative disorders. Yet, a major
drawback is that when applied as a neuroprotective agent,
recombinant EPO present in the blood circulation will also
give rise to an increase of the red blood cells mass or
hematocrit. This, in turn, leads to a higher blood
25   viscosity, which may have detrimental effects in brain
ischemia (Wiessner et al. 2001).

   The present invention provides a solution for the
problem that recombinant human EPO that has been applied
thus far as a neuroprotective agent has the undesired
30   haematotropic side effect (Wiessner et al. 2001). Thus, it
is shown that PER.C6-produced brain-type glycosylated
recombinant human EPO has a high potential as a

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neurogenesis and/or a neuroprotective agent whereas it has a low potential in stimulating erythropoiesis.

According to the invention, PER.C6-produced EPO can be administered systemically (intra-venous, intra-peritoneal,

5    intra-dermal) to inhibit, to prevent and/or to repair the neural damage that is caused by, for example, acute head and brain injury or neuro-degenerative disorders. The present invention also provides products that can be used to modulate the function of tissues that might get heavily

10   damaged by hypoxia, such as the central- and peripheral nervous system, retinal tissue and heart tissue in mammals. Such tissues may be diseased but may also be normal and healthy. Disorders that can be treated by products provided by the present invention may result from acute head-,

15   brain- and/or heart injuries, neuro-degenerative diseases, seizure disorders, neurotoxin poisoning, hypotension, cardiac arrest, radiation, multiple sclerosis and/or from injuries due to hypoxia. Hypoxia may be the result of prenatal- or postnatal oxygen deprivation, suffocation,

20   emphysema, septic shock, cardiac arrest, choking, near drowning, sickle cell crisis, adult respiratory distress syndrome, dysrythmia, nitrogen narcosis, post-surgical cognitive dysfunction, carbon monoxide poisoning, smoke inhalation, chronic obstructive pulmonary disease

25   anaphylactic shock or insulin shock. Seizure injuries include, but are not limited to, epilepsy, chronic seizure disorder or convulsions. In case the pathology is a result from neuro-degenerative diseases the disorder may be due to AIDS dementia, Alzheimer's disease, Parkinson's disease,

30   Creutzfeldt-Jakob disease, stroke, cerebral palsy, spinal cord trauma, brain trauma, age-related loss of cognitive function, amyotrophic lateral sclerosis, alcoholism,

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retinal ischemia, glaucoma, general neural loss, memory loss or aging. Other examples of diseases that may be treated with products provided by the present invention include autism, depression, anxiety disorders, mood

5    disorders, attention deficit hyperactivity disorder (ADHD) and cognitive dysfunction.

PER.C6-EPO can passively cross the blood-brain barrier in case of blood-brain barrier dysfunction. In case the blood-brain barrier is intact, PER.C6-EPO might be actively

10   transported over the blood-brain barrier through the EPO-R. Some studies suggested that EPO in itself is able to cross the blood-brain barrier when high doses of recombinant EPO is administered (WO 00/61164). Another possible route for recombinant PER.C6-EPO to cross the blood-brain barrier is

15   via the interaction of the Lewis x glycan structures present on the PER.C6-produced EPO with E-selectin molecules present on human brain microvessel endothelial cells (Lou et al. 1996). Interaction between E-selectin and EPO may facilitate the transport of EPO across the cerebral

20   endothelial barrier since E-selectin also has been implicated in the migration of T lymphocytes into the CNS (Wong et al. 1999). If required for optimal neuro-protection, PER.C6-produced EPO can be administered at a significantly higher dose than serum-type EPO, because

25   PER.C6-EPO will induce erythropoiesis much less efficiently, such that the detrimental effects of the increase in hematocrit is reduced or even absent.

In another disclosure of the invention, PER.C6-EPO can be administered intrathecally by infusion, or through an

30   indwelling ventricular catheter, or through lumbar injection, to inhibit or prevent neural damage. Again, the advantage of using brain-type EPO over serum-type EPO is

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that in the event of leakage into the blood circulation in the case of blood-brain barrier dysfunction, due to for instance stroke, no undesired side-effects with respect to erythropoiesis will occur.

5       The present invention establishes that indefinitely growing transformed cells that grow to very high densities under serum-free conditions and that have strong neural characteristics, such as PER.C6, are extremely useful to produce factors that depend for their functionality on these characteristics. This inherently also provides the possibility to produce factors that do not have neural features or neural-related functions but that nevertheless benefit from the post-translational modifications that are brought about by such cells. One can envision that some factors also play a role in non-neural tissue but that still require glycosylation structures that include for instance Lewis x structures or fucose residues as described for EPO in the present invention and that can be provided by the means and methods of the present invention. Examples of factors that might be produced by PER.C6 and that take advantage of the neural characteristics of PER.C6 cells include, but are not limited to, brain-type erythropoietin, transferrin and the different factors mentioned above. The invention shows that it is very likely that the production of other recombinant neurotrophic glycoproteins will benefit from the brain-type modifications that take place in such cells.

In accordance with the present invention it has surprisingly been found, that erythropoietin-like molecules having on average a lower sialic acid residue count per protein backbone are still effective in the treatment

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and/or prevention of various disorders. This opens entirely
new ways to use EPO and EPO-like molecules hitherto
believed to of less or no use, including but not limited to
low-sialyl EPO-fractions of EPO batches produced on
5    recombinant mammalian cell systems, discarded upon
fractionation because of their low average sialylation
degree and/or low associated erythropoietic activity.
Thus, the present invention demonstrates that EPO with a
low sialic acid content is about as potent in reducing
10   infarct size in an experimentally induced stroke in rats as
EPO with a higher sialic acid content. It is well
established in the art that a high sialic acid content of
EPO correlates to longer circulatory half-lifes and
increased erythropoietic potential in vivo (Tsuda et al.
15   1990; Morimoto et al. 1996).

        Hence, in general terms, the invention provides the
use of a composition of erythropoietin-like molecules
selected from the group consisting of one or more muteins
of erythropoietin, one or more derivatives of
20   erythropoietin, and a composition of one or more fractions
of erythropoietin molecules sialylated to a varying degree,
for the preparation of a medicament for the treatment of a
disorder selected from the group consisting of ischemia, a
reperfusion injury, a hypoxia-induced disorder, an
25   inflammatory disease, a neurodegenerative disorder, and
acute damage to the central- or peripheral nervous system,
wherein said composition of erythropoietin-like molecules
has on a protein content basis a lower erythropoietic
activity in vivo than epoetin alfa. Embodiments of the
30   invention comprise compositions and use thereof wherein
said erythropoietic activity in vivo is at least 10, 20,
30, 40, 50, 60, 70, 80, or 90% lower than that of epoetin

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alfa (Eprex). Erythropoietin-like molecules are meant to
include molecules that have a protein backbone that is
identical to or similar to the presently known forms of
EPO, e.g. EPO muteins, EPO derivatives, or EPO molecules

5   differing in glycosylation of the protein backbone in
qualitative and/or quantitative respect. Muteins as used
herein are meant to consist of erythropoietin-like
molecules that have one or more mutations in the protein
backbone by deletion, addition, substitution and/or

10  translocation of amino acids relative to the protein
backbone of epoietin alfa and shall include naturally
occurring allelic variants as well as genetically and/or
chemically and/or enzymatically obtained variants. Such
molecules should still be able to confer a functional

15  activity of EPO. They are obtainable using standard
techniques of molecular biology, well known to those of
skill in the art. A derivative as used herein is an
erythropoietin-like molecule that is obtainable from
erythropoietin or epoietin alfa, or any other functional

20  mutein of epoietin alfa by the chemical or enzymatic
modification thereof. Erythropoietic activity as meant
herein is the stimulatory effect of EPO on red blood cell
production in a human or animal subject, as can be measured
by the increase in hematocrit values at a certain point in

25  time after administration to the human or animal subject of
erythropoietin-like molecules (e.g see example 9), or the
measuring the hemoglobin concentration. These methods are
all well known those of skill in the art. Epoetin alfa is
the recombinant human EPO form present in currently

30  marketed Eprex-TM, and is similar or identical (with
respect to amino acid and carbohydrate composition) to
human erythropoietin isolated from urine of anemic

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patients. Treatment regimes for erythropoietic purposes are
well established. In general EPO dosages are given in IU
(international units), referring to the activity of EPO in
erythropoiesis. Such IU correlate to the protein content of
5    EPO but are operationally defined, and hence the
correlation may vary between different batches. As a rule
of thumb, one IU corresponds to 8-10 ng epoetin alfa. For
the purpose of describing the invention the erythropoietic
activity of the erythropoietin-like molecules is referred
10   to on a protein content basis, to get rid of the variable
introduced by defining IU. It will be clear to the person
skilled in the art that although the IU are usually given
for commercial EPO preparations, the concentration of EPO
molecules in such preparations can easily be defined
15   according to standard procedures. This will allow to
determine the relative specific activity e.g in IU/g (see
e.g. EP 0428267). Several *in vivo* and *in vitro* assays
useful for these purposes are also described by Storring et
al. (1992). Examples of other forms of EPO currently on the
20   market are Procrit or Epogen (both epoetin alfa) and
Aranesp (darbepoetin alfa, EPO with extra N-glycosylation
sites to increase circulatory half-life and erythropoietic
activity). Although the erythropoietic activity may vary
somewhat between the various commercial epoetin alfa
25   preparations on the market, they are generally optimized
for high erythropoietic activity. The present invention
discloses the use of EPO-like molecules or EPO-forms that
have a lower hemopoietic or erythropoietic activity,
thereby diminishing or avoiding the side-effects of
30   increased erythropoiesis when this is not desired.
    According to another embodiment of the invention, a
composition of erythropoietin-like molecules is

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characterized by an average number of sialic acid residues
per erytropoetin-like molecule that is at least 10% lower
than the average number of sialic acid residues per
erythropoietin molecule in epoetin alfa. According to other

5    embodiments, said average number of sialic acid residues
may be chosen to be at least 20%, 30%, 40%, 50%, 60%, 70%,
80% or 90% lower than the average number of sialic acid
residues per EPO protein backbone in epoetin alfa. Said
average number of sialic acid residues in the

10   erythropoietin-like molecule preferably lies between
between 0 and 90% of the average number of sialic acid
residues per EPO molecule in epoetin alfa, but the exact
percentage may depend from disorder to disorder, and –
sometimes – from patient to patient, as some patient –

15   disorder combinations are less vulnerable to high
hematocrit values than others. Alternatively, the number of
sialic acid residues could be described per EPO-like
molecule, e.g. 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3,
2, 1 or 0 sialic acid residues per EPO-like molecule. Since

20   the values are averages calculated for a composition that
consists of epo-like molecules of varying degree of
sialylation, non-integer values in between the mentioned
values are possible to define the molecules according to
the invention. The optimal range could be determined

25   empirically. The average number of sialic acid residues per
molecule or the sialic acid content of EPO can be
determined according to published procedures, and are well
known to persons skilled in the art. One possible procedure
is described in EP 0428267. In brief, the sialic acid

30   residues are cleaved from the EPO-like molecules by
hydrolysis with 0.35 M sulfuric acid at 80°C for 30
minutes, and the solutions are neutralized with sodium

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hydroxide prior to analysis. Alternatively, the sialic
acids can be removed by enzymatic cleavage according to
standard procedures. The amount of EPO is estimated using
well known procedures e.g. by using commercially available

5      protein assay kits (e.g. Bradford assay, Biorad) and
standard curves using recombinant human EPO as a standard,
absorbance at 280 nm, ELISA, RIA, and the like. Sialic acid
content can be analyzed by the procedure of Jourdian et al.
(1971). Alternatively, sialic acids can be analysed using

10     High Performance Anion-Exchange Chromatography, using
procedures well known to the skilled person (e.g. Analysis
of Sialic Acids using High-Performance Anion-Exchange
Chromatography, Application note number TN41, Dionex). The
sialic acid content can be expressed as moles of sialic

15     acid per mole of EPO, or an average number of sialic acid
residues per EPO-like molecule. An indication for the
average number of sialic acid residues per EPO-like
molecule can also be given by iso-electric focusing (see
example 4), which measures the pI.

20          Several ways can be envisaged to obtain
erythropoietin-like molecules with an average lower number
of sialic acid residues per erythropoietin-like molecule.
These include, but are not limited to treatment of EPO-like
molecules, e.g.produced recombinantly in any suitable host

25     cell line, using enzymes that cleave off the sialic acid in
particular, such as neuraminidases, or enzymes that cleave
off more substituents (including sialic acid) of the
glycosylation structures, such as e.g. N-glycanase F
(removes whole N-glycan), endoglycosidase $F_2$ (removes bi-

30     antennary structures), endoglycosidase $F_3$ (removes bi- and
tri-antennary structures, and the like, or treatment of
EPO-like molecules with chemicals, including but not

limited to acids, that results in decrease of the average
number of sialic acid residues per EPO-like molecule. In
particular, a highly sialylated EPO fraction could be thus
desialylated and used in the present invention. In yet

5   another embodiment EPO-like molecules with an average lower
number of sialic acid molecules are obtained by purifying
or separating such forms from a mixture containing both
higher and lower sialylated EPO. The currently used
production systems generally result in such mixtures, and

10  EPO that is intended for erythropoietic purposes is
prepared by purifying the forms with a high average number
of sialic acid residues. The present invention discloses
use of other fractions from this process, i.e. the EPO
forms with a lower number of sialic acid residues.

15  Purifying or separating such fractions can be done using
well-established techniques known to the skilled person,
such as ion-exchange, affinity purification, and the like.
The erythropoietin-like molecules of the invention are
preferably produced recombinantly. This can be done in any

20  suitable expression system, including but not limited to
Chinese Hamster Ovary cells, Baby Hamster Kidney cells,
human cells, such as HeLa, HEK293 or PER.C6. Expression in
lower eukaryotic cells such as insect cells or yeast is
also possible. Production of EPO-like molecules having low

25  sialic acid content may be performed on sialylation-
deficient cell systems, by way of a natural lack of
sialylating enzymes, such as certain prokaryotic hosts, or
by mutagenesis or genetic modification of hosts otherwise
capable of producing sialylated proteins. Methods and means

30  to produce recombinant proteins are well documented and
known to the person skilled in the art, and it will be
clear to the skilled person that using a different source

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for the EPO-like protein is possible without departing from
the scope of the invention. In one aspect of the invention,
the EPO-like molecules are produced by methods according to
the invention, thereby producing molecules with a

5      predetermined post-translational modification.
In another aspect of the invention, the composition
comprising erythropoietin-like molecules is characterized
by the presence of erythropoietin-like molecules that once
administered parenterally to a human or an animal subject

10     are cleared from the bloodstream at a faster rate than
epoetin alfa. Clearance from the bloodstream can be
measured by methods well known in the art, e.g. by
determining the half-life of a protein in blood such as
done in example 18. In healthy volunteers epoetin alfa has

15     a circulatory half-life of about 4 hours after repeated
intravenous injections. A half-life of about 5 hours in
patients with chronic renal insufficiency, and about 6
hours in children has been reported. Using the method of
example 8, we measure a half-life of 180 min for epoetin

20     alfa (Eprex). It should be clear to the skilled person that
this method can be used to determine the half-life of the
compositions of the invention, and express this half-life
in hours or in a percentage of the half-life of the
standard EPO (Eprex). Similar experiments are feasible in

25     humans to determine the half-life in humans.
Erythropoietin-like molecules with a lower ratio of tetra-
antennary structures to bi-antennary structures will also
have a shorter half life in plasma (Misaizu et al, 1995;
Takeuchi et al, 1989). Production of EPO in cell lines that

30     give rise to such lower ratios is feasible, or
alternatively these forms are purified away from the forms
containing more tetra-antennary structures. Such

compositions comprising relatively more bi-antennary
structures are also useful according to the invention. It
wil also be clear that one advantage of the current
invention is that higher maximal concentrations of

5    erythropoietin-like molecules in the circulation can be
reached as compared to the currently used EPO forms such as
Eprex, Procrit, NESP. If high concentrations of EPO-like
molecules would be desired for said treatment, this can be
done by administering high doses of the compositions of the

10   invention. Administering of similar doses on a protein
content basis of the currently used EPO-like molecules
would lead to higher erythropoiesis, which is an undesired
side-effect for said treatments.
The invention also provides pharmaceutical compositions

15   comprising said erythropoietin-like molecules, and methods
for treatment or preventing disorders selected from said
groups, as wel as compositions of erythropoietin-like
molecules for for the preventative and/or therapeutic
treatment of the human or animal body.

EXAMPLES


**Example 1. Studies on expression of marker proteins in PER.C6™ cells.**

5       The cells that were transformed with the E1 region of
human Adenovirus type 5 and that resulted in the PER.C6™
cell line (as deposited under ECACC no. 96022940) were
derived from a human embryonic retina. Retinas generally
comprise a number of different cells types (at least 55
10    different neural subtypes), including neural and
fibroblast-like cells (Masland 2001). In order to trace the
cellular origin of PER.C6, a study was performed to test
the expression of marker proteins in or on the cells. These
markers are known in the art to be characteristic for
15    certain cell types and/or tissues. The marker proteins are
given in Table I.
        Marker protein expression was tested using antibodies
directed against the marker proteins. In each experiment, a
negative control (PER.C6 cells not incubated with antibody)
20    and a positive control were taken along. These positive
controls are sections of human tissue known to express the
marker protein (Table II).
        PER.C6 cells were cultured on glass slides in a medium
chamber (Life Technologies, Nunc Lab-Tek, Chamber Slide,
25    radiation sterilized, 2 medium chambers, cat.no. 154464A).
PER.C6 cells were seeded at 65-70% confluency (2 wells per
culturing chamber) and cultured for 24 h at 37°C (10% $CO_2$,
95% air). The medium was aspirated and the glass slides
with cells were washed with sterile PBS, removed from the
30    medium chamber and air-dried. Cells were fixed on the glass
slides by incubation in acetone for 2 min. After air

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drying, slides were wrapped in aluminum foil and frozen at
a temperature lower than -18°C until use.

Positive control tissues were obtained from banks of
tissue slides prepared for routine use at the division of
5    pathology, Academic Hospital Erasmus University (Rotterdam,
The Netherlands). Frozen sections were prepared (5 µm) and
fixed in acetone, according to routine procedures.

The primary antibodies, their respective marker
proteins, the suppliers and the catalog numbers of the
10   antibodies are given in Table III. The dilutions, also
detailed in Table III, are made in Phosphate Buffered
Saline (PBS), 1% Bovine Serum Albumin. Incubations of the
slides with the primary antibody were done for 30 min at
room temperature, rinsed with PBS and incubated with the
15   secondary antibody. These secondary antibodies were either
goat anti rabbit (DAKO E0432; 1:50 dilution) or goat anti
mouse (DAKO E0433; 1:50 dilution), depending on the nature
of the primary antibody used. The second antibody was
conjugated with biotin. After rinsing with PBS, the slides
20   were incubated with streptavidin-avidin/biotin complex
conjugated with alkaline phosphatase (DAKO, K0376). After
30 min of incubation, the samples were rinsed with Tris/HCl
pH 8.0, developed with fuchsin substrate chromagen (DAKO
K0624) in the dark room for 30 min. Subsequently, the
25   slides were rinsed with tap water for 2 min and
counterstained with hematoxylin according to routine
procedures well known to persons skilled in the art. Then,
the slides were examined microscopically and scored for
marker protein expression (negative or positive). The
30   results are presented in Table IV. For neurofilament
staining (positive) not all PER.C6 cells did stain positive
as a result of a different cell cycle- or maturation phase

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of the cell population. This is a normal observation for
neurofilament stainings.

From the data obtained it was concluded that PER.C6
cells are of neural origin since the cells stained positive

5     for vimentin, synaptophysin, neurofilament, GFAP and N-CAM.


**Example 2. Monosaccharide composition of PER.C6-EPO derived**
**N-glycans compared to that of Eprex.**

A first step in characterizing the N-glycan structures

10    produced by PER.C6 is the measurement of the molar ratio of
the various monosaccharides. The monosaccharide analysis
was performed using high performance anion exchange
chromatography with pulsed amperometric detection (HPAEC-
PAD). EPO samples, produced by PER.C6-derived clones P7,

15    P8, and C25 (P7 and P8 are described in WO 00/63403, and
C25 was generated generally according to these methods,
using Neomycin resistance gene as a selection marker) in
DMEM and/or JRH medium, were selected for this analysis.
Eprex (Jansen Cilag), which is the commercially available

20    recombinant CHO-derived erythropoietin, was analyzed in
parallel, and therefore used as a reference.

PER.C6-EPO samples were purified by affinity
chromatography using a column packed with C4 sepharose
beads (bedvolume of 4 ml, Amersham Pharmacia Biotech)

25    coupled with mouse monoclonal anti-EPO (IgG1) antibodies.
Bound EPO molecules were eluted with 0.1 M glycine-HCl, pH
2.7, and resulting fractions were immediately neutralized
by adding sodium/potassium phosphate buffer pH 8.0.
Subsequently, the fractions containing EPO were pooled and

30    the buffer was exchanged to 20 mM Tris-HCl, containing 0.1%
(v/v) Tween 20, by utilizing Hiprep 26/10 desalting columns
(Amersham Pharmacia Biotech).

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For glycan analyses, purified EPO samples were
dialyzed overnight against MilliQ-grade water, and dried in
a Speedvac evaporator. Dried EPO samples (quantities ranged
from 39 to 105 µg) were dissolved in incubation buffer (1:1
5    diluted C3 profiling buffer, Glyko). Upon addition of
sodium dodecyl sulfate (SDS) and beta-mercaptoethanol to
final concentrations of 0.1% (w/v) and 0.3% (v/v),
respectively, samples were denatured for 5 min at 100°C.
Nonidet P-40 (BDH) was thereafter added to a final
10   concentration of 0.75% (v/v), and EPO was deglycosylated
overnight at 37°C, using *N*-glycanase F (mU, Glyko). Upon
deglycosylation, released N-glycans were separated from
proteins, salts, and detergents by using graphitized carbon
black (Carbograph) SPE columns (Alltech), according to
15   Packer et al. (1998).

Purified N-glycan chains were subjected to hydrolysis
in 2 M trifluoroacetic acid (TFA) at 100°C for 4 h. After
hydrolysis, monosaccharides were dried in a Speedvac
evaporator, washed with water, and again evaporated in a
20   Speedvac. Dried monosaccharides were dissolved in 26 µl
MilliQ-grade water. After addition of 6 µl deoxyglucose
(100 nmol/ml), which was used as internal standard, samples
(24.5 µl) were applied to an HPAEC-PAD BioLC system with a
2 mm-diameter CarboPac PA1 column (Dionex). The column was
25   run isocratically in 16 mM NaOH (Baker) at a flow rate of
0.25 ml/min. The monosaccharide composition was calculated
by comparing the profile with that obtained with a mixture
of monosaccharide standards that consisted of fucose,
deoxyglucose, galactosamine, glucosamine, galactose, and
30   mannose.

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The monosaccharide analysis clearly showed that the glycosylation status of PER.C6-EPO is significantly different from Eprex (Table V). The ratio of the indicated monosaccharides (Man = mannose, Fuc = fucose, GalNAc = N-

5  acetyl-galactosamine, GlcNAc = N-acetyl-glucosamine, Gal = galactose) was normalized to 3 Man. The duplo values are given between brackets. Interestingly, the PER.C6-EPO samples contain significant amounts of GalNAc, whereas the N-linked sugars of Eprex lack this residue. This suggests

10  that PER.C6-EPO contains so-called LacdiNAc (e.g., GalNAcβ1-4GlcNAc) structures. Another striking feature of PER.C6-EPO is the relative abundance of fucose residues shown in Table V. This strongly indicates the presence of Lewis structures in the N-glycans of PER.C6-EPO. In

15  contrast, Eprex is known to be devoid of Lewis structures. Consequently, the amount of fucose found in Eprex can be solely attributed to N-glycan core fucosylation. Notably, the data from the monosaccharide analyses also demonstrated that culture conditions affect the glycosylation status of

20  EPO in PER.C6. It should not be concluded that the culture conditions are solely responsible for the predetermined post-translational modifications that are present on the proteins produced. Of course the cell lines should be able to modify the post-translational modifications of the

25  proteins produced on such cells through the presence of certain specific glycosylation enzymes such as transferases. The culture conditions can only exert additive activities. For instance, when the EPO-producing clones were cultured (in suspension) in JRH Excell 525

30  medium, the N-linked glycans of EPO were found to contain higher levels of GlcNAc, GalNAc, Gal, and Fuc as compared to the N-linked sugars of EPO derived from cultured

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(adherent) cells in DMEM (Table V). This effect was
particularly evident in the case of clone P8. The elevated
level of GlcNAc may suggest that the branching of the N-
linked sugars is increased and/or that the N-linked sugars

5    contain more lactosamine repeats, when cells are cultured
in JRH medium. The increase in N-acetyl glucosaminylation
and in (N-acetyl-) galactosylation in turn gives rise to an
increased number of fucose-acceptor sites thereby providing
an explanation for the increase of the Fuc content.

10

**Example 3. Mass spectrometric analysis to reveal structural
differences between N-glycans of PER.C6-EPO and Eprex.**

To obtain more detailed information on the structure
of the N-glycans produced by PER.C6, it was decided to

15   analyze the complete sugar chains of PER.C6-EPO by MALDI-
MS. For this analysis, affinity-purified EPO samples, made
by PER.C6-derived clones P7 and P8 in DMEM, which were
fractionated further by anion exchange chromatography (as
described below) were utilized. PER.C6-EPO samples,

20   affinity-purified as described in example 2, of which the
buffer was thereafter exchanged to PBS, were subjected to
anion exchange chromatography using a HiTrap sepharose Q HP
column (Amersham Pharmacia Biotech). Three EPO subfractions
were obtained by applying a step gradient in 20 mM Tris-

25   HCl/20 µM CuSO$_4$, beginning with 45 mM NaCl (fraction 1),
followed by 75 mM NaCl (fraction 2), and ending with 135 mM
NaCl (fraction 3). Each step of the gradient lasted 10 min
with a flow rate of 1 ml/min. Fractions 1 of four runs were
pooled into pool A, fractions 2 into pool B, and fractions

30   3 into pool C. The resulting pools A, B, and C were
thereafter desalted utilizing HiPrep 26/10 desalting
columns (Amersham Pharmacia Biotech). The N-linked glycans

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were released from the EPO pools by N-glycanase F treatment
and desialylated by neuraminidase treatment. Eprex was
analyzed in parallel as a reference. Representative mass
spectra of the various EPO samples are shown in Fig. 1A-G:

5    Eprex and the purified, fractionated (pools A, B, and C
from the anion exchange chromatography column). PER.C6-EPO
samples derived from the indicated clones cultured in DMEM
were treated with glycanase F and neuraminidase, and
thereafter analyzed by MALDI-MS. Symbols (depicted in the

10   spectrum of Eprex) are: closed square is GlcNAc, open
circle is Man, closed circle is Gal, open triangle is Fuc.
The mass profile of the N-linked sugars of Eprex (Fig. 1A)
corresponds to previously published data and indicates that
tetra-antennary sugars with or without lactosamine repeats

15   predominate in this EPO preparation. Although Eprex and
PER.C6-EPO contain sugar structures with a similar mass
(Fig. 1B-G), the profile of the sugar structures of the
latter is much more complex, suggesting that these sugars
display a large degree of heterogeneity. The ExPASy's

20   computer program was used to predict the sugar composition
on basis of the observed mass (Table VI and VII). The
relative abundance of the different oligo-saccharides in
each pool was also presented. Strikingly, the data
demonstrated that most N-linked oligosaccharides derived

25   from PER.C6-EPO contain multiple fucose residues (Table VI
and VII, see level of dHex residues). Some glycans were
even quadruple-fucosylated. Consequently, these data are in
line with our monosaccharide analyses and strongly suggest
that PER.C6-EPO is hyperfucosylated, and, hence, most

30   likely decorated extensively with N-glycans having so-
called Lewis structures. Oligosaccharides with (sialylated)
Lewis x epitopes are known as essential recognition

sequences for selectins, mediating cell-cell adhesions in
both inflammatory and immune responses (Varki et al. 1999)
and are characteristically found in brain glycoproteins
(Margolis and Margolis 1989). Hence, numerous glycoproteins

5   carrying these Lewis x structures have been shown to have
therapeutic potential by exhibiting anti-inflammatory and
immunosuppressive activities. It is emphasized here that a
mass signal cannot always be unambiguously assigned to a
certain sugar structure, as e.g. residues, like GlcNAc and

10  GalNAc, have the same mass. Because the monosaccharide
analysis of PER.C6-EPO revealed the occurrence of GalNAc in
the N-linked sugars, it is expected that some of the peaks
represent N-glycans with so-called LacdiNAc (e.g.,
GalNAc$\beta$1-4GlcNAc) structures. For example, peaks with $m/z$

15  values of ~ 2038 and ~ 2185 (Table VI and VII) most likely
represent N-glycans with LacdiNAc motifs. Otherwise, these
peaks would represent tetra-antennary structures, which
terminate in GlcNAc due to the absence of Gal or GalNAc.
Although such structures may be present due to incomplete

20  glycosylation, the presence of the proximal Fuc implies
that the sugar contained a Gal or GalNAc residue that is
necessary to form a motif that is recognized by the
fucosyltransferase (FUT) that catalyzes the formation of
the Lewis structure.

25        The relative occurrence of the different sugars varies
between the EPO preparations derived from two independent
PER.C6 clones as judged by the difference in the relative
height of certain peaks. In particular, the putative bi-
antennary sugars with LacdiNAc motifs (Fig. 1; Table VI and

30  VII, signals with $m/z$ values of ~ 2038 and ~ 2185) are
clearly the major sugars in EPO samples derived from P8,

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whereas in P7 samples these structures are far less
abundant. In the latter clone, the peak with an *m/z* value
of ~ 2541, putatively corresponding to a fully
galactosylated tetra-antennary glycan, was the most
5    abundant structure. These data are in accordance with our
monosaccharide analyses, which already indicated that, when
grown in DMEM, P8 produced EPO carrying glycans with a
lower degree of branching than those derived from P7-EPO
(Table V).

10

**Example 4. Comparison of sialic acid content of PER.C6-EPO
and CHO-EPO.**

The sialic acid content of PER.C6-EPO was analyzed and
compared with erythropoietin derived from Chinese Hamster
15   Ovary cells (CHO-EPO) by iso-electric focusing (IEF) using
IPG strips (Amersham Pharmacia Biotech) that have a linear
pH gradient of 3-10. After the focusing, the EPO isoforms
were passively blotted onto nitrocellulose, and visualized
using an EPO-specific antibody and ECL (Fig. 2). EPO made
20   by four different PER.C6 clones (lanes C, D, E, and F), and
three different CHO clones stably expressing EPO (lanes G,
H, and I) were analyzed by iso-electric focusing to
determine the sialic acid content. The EPO producing CHO
and PER.C6 cell lines were generated generally according to
25   methods described in WO 00/63403 using the Neomycine-
resistance gene as a selection marker. One thousand eU of
PER.C6-EPO and 500 eU of CHO-EPO were loaded per strip.
Five hundred IU of Eprex (lane A) and neuraminidase-treated
(partially desialylated) Eprex (lane B) were used to
30   identify the various EPO isoforms. After focusing, EPO was
blotted onto nitrocellulose filter and visualized using a

monoclonal antibody against EPO and ECL. The Eprex sample,
representing a commercially available EPO is a formulation
containing highly sialylated isoforms and was used as a
marker.

5          The results clearly demonstrated that CHO cells are
able to make EPO isoforms containing up to at least 12
sialic acids per molecule (lanes G-I), confirming data by
Morimoto et al. (1996). In contrast, although some isoforms
with 8-10 sialic acids were produced by PER.C6, these were
10    obviously underrepresented and only detectable after
prolonged exposure of the film (lanes C-F). Consequently,
it can be concluded that PER.C6-EPO is considerably less
sialylated than CHO-EPO.


15    **Example 5. α1,3-, α1,6- and α1,2-fucosyltransferase
activities on PER.C6 cells.**

          The glycosylation potential of a cell is largely
determined by an extensive repertoire of glycosyl-
transferases involved in the step-wise biosynthesis of N-
20    and O-linked sugars. The activity of these glycosyl-
transferases varies between cell lines and, hence,
glycoproteins produced in different cell lines acquire
different glycans. In view of the data shown herein,
demonstrating that PER.C6-EPO glycans are heavily
25    fucosylated, the activity of numerous fucosyltransferases
(FUTs) involved in the synthesis of N-linked sugars were
analyzed using methods generally known to persons skilled
in the art (Van den Nieuwenhof et al. 2000). In this study,
we studied the activities of α1,6-FUT, which is involved in
30    core fucosylation of N-glycans, α1,2-FUT which mediates the
capping of terminal galactose residues, giving rise to so-
called Lewis y epitopes, and α1,3-FUT, which generates

Lewis x structures. For comparison, we also analyzed the corresponding FUT activities present in CHO cells.

The activities of the indicated FUTs in cell-extracts of PER.C6 and CHO were measured using a glycosyltransferase
5   activity assay. This assay measures the glycosyltrans-ferase-catalyzed reaction between a saccharide (in this case fucose) and a sugar substrate. The GalT activity was also measured as an internal control. The values represent the mean values from two experiments. All values, and in
10  particular those of PER.C6 were 2-3 fold lower in the second experiment. Notably, the activities were expressed per mg protein (present in the cell extract). Because PER.C6 cells are significantly bigger than CHO cells, the differences between the FUT and GalT activities of CHO and
15  PER.C6 cells may be bigger or smaller than they appear. The results of the glycosyltransferase activity assays are shown in Table VIII and reveal that PER.C6 as well as CHO possess significant $\alpha 1,6$-FUT activity, which suggests that both cell lines can produce core-fucosylated glycan chains.
20  $\alpha 1,3$-FUT activity was, however, only significant in PER.C6 cells while hardly detectable in CHO cells. None of the two cell lines exhibited $\alpha 1,2$-FUT activity. Taken together, these data show a clear difference between the glycosylation potential of CHO and PER.C6, and explain why
25  PER.C6-EPO contains more fucoses than CHO-produced EPO (Eprex).

**Example 6. Glycans with Lewis x epitopes present on PER.C6-EPO.**

30  Because PER.C6 possesses $\alpha 1,3$-, but no $\alpha 1,2$-fucosyltransferase activity, it is very likely that PER.C6 produced N-glycan chains which contain Lewis x instead of

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Lewis y epitopes. We verified this by labeling PER.C6-EPO
with a mouse monoclonal antibody (Calbiochem) that
specifically recognizes Lewis x structures, using western
blotting. Equal amounts of PER.C6-EPO (derived from clone
5     P7, here indicated as P7.100) and Eprex, untreated (-) or
treated with HCl (+), were run on a SDS-polyacrylamide gel
and blotted onto a nitrocellulose membrane using methods
known to persons skilled in the art. A monoclonal antibody
(anti-mouse IgM, Calbiochem) and ECL (Amersham Pharmacia
10    Biotech) were used to detect the Lewis x epitope. As can be
seen in Fig. 3, only PER.C6-EPO could be labeled with the
antibody specific for the Lewis x epitope. Location of the
molecular weight marker (52, 35 and 29 kDa) is indicated.
Because the $\alpha 1,3$-fucose linkage is acid-labile, the signal
15    was lost after treatment with HCl. Yet, it has to be noted
that the acid treatment also destroyed part of the EPO
since a reduced signal was observed when the blot was
probed with an EPO-specific antibody (not shown).

20    **Example 7. Lewis x structures expression at cell surface of
PER.C6 cells.**

      To find out whether Lewis x structures generally occur
in PER.C6 cells, we labeled the surface of CHO and normal
(i.e., not EPO producing) PER.C6 cells with Lewis x
25    specific antibodies (Calbiochem). The cells were incubated
with the primary antibodies (mAb $\alpha$ Lewis x used at 0.16
µg/ml, and mAb $\alpha$ sialyl-Lewis x used at 5 µg/ml). FITC-
conjugated anti-IgM was used as a secondary antibody. The
labeled cells were analyzed by FACS. The dashed line
30    represents the signal of cells incubated with the secondary
antibody only (negative control). The results shown in Fig.
4 revealed that PER.C6 cells were strongly labeled with the

antibodies in contrast to CHO cells that are unable to produce these structures. Notably, we repeatedly observed that PER.C6 cells displayed a heterogeneous pattern of staining with the Lewis x antibodies. Labeling with an

5   antibody specific for sialyl Lewis x structures (Calbiochem) gave a moderate positive signal only when a very high concentration of the antibody was used.

**Example 8. Inhibition of apoptosis by PER.C6-EPO (brain-**

10  **type) *in vitro*, in NT2 cells and hNT cells cultured under hypoxic conditions.**

PER.C6-produced (brain-type) EPO and serum-type EPO are compared in their *in vitro* activity to protect rat-, mouse- and human cortical neural cells from cell

15  death under hypoxic conditions and with glucose deprivation. For this, neural cell cultures are prepared from rat embryos as described by others (Koretz et al. 1994; Nagayama et al. 1999; White et al. 1996). To evaluate the effects of PER.C6-produced brain-type EPO and serum-

20  type EPO, the cells are maintained in modular incubator chambers in a water-jacketed incubator for up to 48 h at 37°C, in serum-free medium with 30 mM glucose and humidified 95% air/5% $CO_2$ (normoxia) or in serum-free medium without glucose and humidified 95% $N_2$/5% $CO_2$ (hypoxia and glucose

25  deprivation), in the absence or presence of 30 pM purified PER.C6-produced brain-type EPO or 30 pM Eprex. The cell cultures are exposed to hypoxia and glucose deprivation for less than 24 h and thereafter returned to normoxic conditions for the remainder of 24 h. The cytotoxity is

30  analyzed by the fluorescence of Alamar blue, which reports cells viability as a function of metabolic activity.

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In another method, the neural cell cultures are
exposed for 24 h to 1 mM L-glutamate or α-amino-3-hydroxy-5-
methylisoxazole-4-propionic acid (AMPA) under normoxic
conditions, in the absence or presence of various
5    concentrations of purified PER.C6-produced EPO or Eprex.
The cytotoxity is analyzed by the fluorescence of Alamar
blue, which reports cell-viability as a function of
metabolic activity. The viability of cells treated with
PER.C6-EPO is expected to be similar to the viability of
10   cells treated with Eprex.


**Example 9. Activity of PER.C6-EPO (brain-type) in
stimulating erythropoiesis in rats compared to serum-type
EPO.**

15       The potential of recombinant human EPO to stimulate
the production of red blood cells can be monitored in a
rodent model that has been described by Barbone et al.
(1994). According to this model, the increase in the
reticulocyte counts is used as a measure for the biological
20   activity of the recombinant human EPO preparation.
Reticulocytes are the precursors of red blood cells and
their production, in response to EPO, can be used as a
measure for the potential of EPO in stimulating the
production of red blood cells. An increased production of
25   red blood cells, in turn, leads to a higher hematocrit
value.
         The activities of PER.C6-EPO and Eprex were compared
in six groups of three Wag/Rij rats. Various doses of
PER.C6-EPO (P7-EPO), Eprex and diluent buffer as a negative
30   control were injected intravenously in the penile vein at
day 0, 1, and 2. PER.C6-EPO was administered at a dose of
5, 25, or 125 eU (Elisa units) as determined by the

commercially available EPO-specific R&D Elisa Kit, whereas
Eprex was administered at a dose of 1 or 5 eU. All EPO
preparations were diluted to the proper concentration in
PBS/0.05% Tween 80 in a total volume of 500 μl. At day 3,
5    250 μl of EDTA blood was sampled by tongue puncture. On the
same day, the percentage of reticulocytes in the total red
blood cell population was determined.

       As shown in Fig. 6 (bars indicate the percentage of
reticulocytes present in the total red blood cell
10   population), the daily administration of 1 eU of Eprex into
the rats, for a total period of three days, caused a
significant increase in the reticulocyte counts at the
fourth day compared to reticulocyte counts in rats that
received diluent buffer only. The reticulocyte counts were
15   even more boosted by increasing the Eprex dose five-fold.
The reticulocyte counts were clearly less increased using
equivalent amounts of PER.C6-EPO. A similar increase in
reticulocyte counts was observed when 1 eU of Eprex and 25
eU of PER.C6-EPO was used indicating that PER.C6-EPO is at
20   least 25 times less active in stimulating the red blood
cell production than Eprex. The difference between the
potential of Eprex and PER.C6-EPO in stimulating the red
blood cell production was even more pronounced at a higher
dose (i.e. 5 eU Eprex and 125 eU PER.C6-EPO).
25

**Example 10. Effect of PER.C6-EPO on cerebral ischemia
following experiment subarachnoid hemorrhage.**
       To show that PER.C6-EPO is more effective in neuro-
protection during cerebral ischemia than serum-type EPO, we
30   compare the effects of systemic adminstration of PER.C6-
produced brain-type EPO and serum-type EPO in a rabbit
model of subarachnoid hemorrhage-induced acute cerebral

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ischemia. Therefore, 32 animals that are divided into 4 groups (n=8) are studied.

      Group 1, subarachnoid hemorrhage;

5      Group 2, subarachnoid hemorrhage plus placebo;

      Group 3, subarachnoid hemorrhage plus recombinant human serum-type EPO; and

      Group 4, subarachnoid hemorrhage plus recombinant PER.C6-produced EPO.

10

The experimental subarachnoid hemorrhage is produced by a percutaneous injection of autologous blood into the cisterna magna after anesthesizing the animal. After the injection, the rabbits are positioned in ventral recumbence

15 for 15 min to allow ventral blood-clot formation. Animals of group 2, 3, and 4 are injected with diluent buffer, Eprex, and purified PER.C6-produced brain-type EPO, respectively, at 5 min after the induction of subarachnoid hemorrhage, and are continued at 8, 16, and 24 h

20 thereafter. All injections are administered intra-peritoneally. The diluent buffer consists of serum albumin (2.5 mg/ml), sodium chloride (5.84 mg/ml), anhydrous citric acid (0.057 mg/ml, $H_2O$). The animals are euthanized at 24 h after the subarachnoid hemorrhage, and their brains are

25 removed. The brains are thereafter coronally sectioned at 10-25 μm in a freezing microtome, starting at the bregma and continuing posteriorly to include the cerebellum (Ireland and MacLeod 1993). To visualize and assess the number of ischemia-induced damaged neurons, the slices are

30 stained with hematoxylin and eosin. The number of eosinophilic neuronal profiles containing pyknotic nuclei, per high-power microscopic field (100x) is determined in

five randomly selected sections of the lateral cortex
obtained at several coronal levels posterior to the bregma.
PER.C6-EPO treated animals are expected to have a lower
number of damaged neurons than animals that are not treated
5    or that are treated with a placebo.


**Example 11. Erythropoietin receptor expression in rat
neonatal cardiomyocytes following hypoxia/reoxygenation.**

Primary cultures of neonatal rat cardiomyocytes are
10   prepared from the ventricles of 1-day-old Sprague-Dawley
rats, as previously described (Simpson and Savion 1982).
Hypoxia was created by incubating the cardiomyoctes in an
airtight Plexiglas chamber with < 1% $O_2$ and 5% $CO_2$/95% $N_2$ at
37°C for 2 h using Gas Pak Plus (BBL). By replacing the
15   medium saturated with 95% air and 5% $CO_2$, the cells were
exposed to normotoxic atmosphere (reoxygenation).

Cardiomyoctes are washed twice with ice-cold PBS and
total RNA is isolated using Trizol (GIBCO), extracted by
chloroform and precipitated by isopropyl alcohol. For
20   Northern analysis, 15 μg of total RNA is separated on a
1.5% formaldehyde/MOPS-agarose gel, blotted to
nitrocellulose, and hybridized with a $^{32}$P-labeled probe for
EPO receptor (± 400 bp cDNA fragment). Hybridization takes
place overnight at 65°C in phosphate buffer, pH 7.2 and is
25   followed by 2 washes in 2xSSC at room temperature, 2 washes
in 0.2xSSC/0.1%SDS at 65°C and 2 washes in 2xSSC at room
temperature. Hybridization signals are visualized by
exposing the membrane to an X-ray film (Kodak). Expression
levels are corrected for GAPDH mRNA levels.

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## Example 12. The effect of brain-type PER.C6-EPO and serum-type EPO (Eprex) on apoptosis in rat neonatal cardiomyocytes, cultured under hypoxic conditions.

Primary cultures cultures of neonatal rat
5   cardiomyocytes are prepared from the ventricles of 1-day-
old Sprague-Dawley rats as previously described (Simpson
and Savion 1982). Hypoxia is created by incubating the
cardiomyoctes in an airtight Plexiglas chamber with < 1% $O_2$
and 5% $CO_2$/95% $N_2$ at 37°C for 2 h using Gas Pak Plus (BBL).
10  By replacing the medium saturated with 95% air and 5% $CO_2$,
the cells are exposed to normotoxic atmosphere
(reoxygenation). The experiment is divided into 4 groups:
    A)  cardiomyocytes cultured under normoxic conditions (95%
        air/5% $CO_2$);
15    B)  cardiomyocytes cultured under hypoxia/reoxygenation
        conditions in the presence of 30 pM purified PER.C6-
        produced EPO;
    C)  cardiomyocytes cultured under hypoxia/reoxygenation
        conditions in the presence of 30 pM purified Eprex;
20      and
    D)  cardiomyocytes cultured under hypoxia/reoxygenation
        conditions in the absence of EPO.
All experiments are performed in triplicate. Apoptosis is
quantified by morphological analysis, DNA laddering and by
25  terminal deoxyribonucleotide transferase-mediated dUTP nick
end labeling (TUNEL). For morphological analysis myocytes
monolayers are fixed and stained with Hoechst 33324. The
morphological features of apoptosis (cell shrinkage,
chromatin condensation, and fragmentation) are monitored by
30  fluorescence microscopy. At least 400 cells from 12
randomly selected fields per dish are counted.

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For determining DNA laddering (characteristic for apoptosis), cardiomyocytes are lysed in lysis buffer and electrophoresed on 2% agarose gel. The gel is stained with ethidium bromide, and DNA fragments are visualized under
5  ultraviolet light. *In situ* detection of apoptotic cardiomyocytes is performed by using TUNEL with an in situ cell death detection kit (Boehringer Mannheim).

**Example 13. The effect of PER.C6-EPO and serum-EPO on the**
10  **infarct size in a rat model of myocardial ischemia/**
**reperfusion.**

Adult male Sprague-Dawley rats (300 to 400 g) are anesthetized with sodium pentobarbital (20 mg/kg IP) and ketamine HCl (60 mg/kg IP). Jugular vein and trachea are
15  cannulated, and ventilation is maintained with 100% oxygen by a rodent ventilator adjusted to maintain exhaled $CO_2$ between 3.5% and 5%. A left thoracotomy was performed and a suture was placed 3 to 4 mm from the origin of the left coronary artery. Five minutes before ischemia animals are
20  randomly given various concentrations of PER.C6-EPO, serum-type EPO or saline (n=6 for each group). Ischemia (30 min) is initiated by tightening of the suture around the coronary artery and is followed by 4 h of reperfusion. Sham-operated rats are prepared identically, except that
25  the suture is not tightened (n=6).

After reperfusion, infarct size is determined by differential staining with patent blue violet (5%) and triphenyl tetrazolium chloride (TTC). The coronary ligature is retightened, and an intravenous injection of patent blue
30  violet is given to stain the normally perfused regions of the heart. The heart is then removed and bathed in ice-cold saline before removal of the atria, great vessels and right

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ventricle. The left ventricle is sliced into thin sections, and the unstained area at risk (AAR) is separated from the normally perfused blue sections, cut into 1-2 mm$^3$ pieces, and incubated with TTC. With a dissecting microscope, the

5    necrotic areas (AN, pale) are separated from the TTC-positive (brick red-staining) areas. All areas of the myocardium are then weighed individually, and infarct size is calculated.

10   **Example 14. Isolation and fractionation of PER.C6-EPO glycoforms employing a high α1,3-linked fucose content.**

The fucose-specific *Aleuria aurantia* lectin (AAL) is used to preferentially purify PER.C6-EPO glycoforms with a high Lewis x and/or sialyl-Lewis x content. This lectin is

15   coupled to CNBr-activated Sepharose 4B beads according to procedures commonly known by a person skilled in the art. PER.C6-EPO that is secreted into the culture medium by human EPO-producing PER.C6 cells is first roughly separated from cell debris and other contaminants by affinity column

20   chromatography using monoclonal antibodies specific for human EPO. Thereafter, the purified EPO is subjected to a second chromatography procedure in which the EPO molecules possessing α1,3-linked fucose are bound to a column containing the immobilized AAL. EPO glycoforms that lack

25   α1,3-linked fucose do not bind to the column and are collected in the flow-through. EPO glycoforms carrying α1,3-linked fucose are eluted from the column by using fucose as a competitor for binding to AAL. EPO glycoforms having a high or low α1,3-linked fucose content are

30   separately eluted from the column by increasing the fucose concentration step-wise or gradually during the elution.

EPO glycoforms with a high α1,3-linked fucose content are eluted at a higher concentration of fucose than EPO glycoforms with a low α1,3-linked fucose content. This method enables one to purify erythropoietin from the

5    culture medium by employing the specific characteristics of the post-translational modifications, such as Lewis x structures brought about by the cells in which the protein is produced.

10    **Example 15. Isolation and fractionation of PER.C6-EPO glycoforms with a high LacdiNAc content.**

PER.C6-EPO glycoforms carrying so-called lacdiNAc oligosaccharide structures are specifically isolated by the use of monoclonal antibodies against these lacdiNAc

15    structures. Mouse monoclonal antibodies such as 99-2A5-B, 100-2H5-A, 114-2H12-C, 259-2A1, and 273-3F2 (Van Remoortere et al. 2000) specifically recognize lacdiNAc structures and are purified and coupled to CNBr-activated Sepharose 4B beads according to procedures commonly known by a person

20    skilled in the art. PER.C6-EPO that is secreted into the culture medium by human EPO-producing PER.C6 cells is first roughly separated from cell debris and other contaminants by affinity column chromatography using monoclonal antibodies specific for human EPO. Thereafter, the purified

25    EPO is subjected to a second chromatography procedure in which the EPO molecules carrying lacdiNAc structures are bound to a column containing the immobilized lacdiNAc-specific monoclonal antibodies. EPO glycoforms that lack the lacdiNAc structures do not bind to the column and are

30    collected in the flow-through. EPO glycoforms carrying the lacdiNAc structures are eluted from the column at a low pH or by using GalNAc or synthetic lacdiNAc oligosaccharides

as a competitor for binding to the lacdiNAc specific
antibodies. EPO glycoforms carrying a relatively high
percentage of lacdiNAc structures are separately eluted
from the column by increasing the GalNAc or lacdiNAc
5   concentration step-wise or gradually during the elution.
EPO glycoforms with a relatively high percentage of
lacdiNAc structures are eluted at a higher concentration of
GalNAc or lacdiNAc than EPO glycoforms possessing a
relatively low percentage of lacdiNac structures. In
10  accordance with the method described above, also this
method enables one to purify erythropoietin from the
culture medium by employing the specific characteristics of
the post-translational modifications, such as Lewis x and
lacdiNac structures brought about by the cells in which the
15  protein is produced.


**Example 16. Isolation and fractionation of PER.C6-EPO
glycoforms with a high GalNAc-Lewis x content.**

        PER.C6-EPO glycoforms carrying so-called GalNAc-Lewis
20  x oligosaccharide structures are specifically isolated by
the use of monoclonal antibodies against these GalNAc-Lewis
x structures. Mouse monoclonal antibodies such as 114-5B1-
A, 176-3A7, 290-2D9-A, and 290-4A8 (Van Remoortere et al.
2000) specifically recognize GalNAc-Lewis x structures and
25  are purified and coupled to CNBr-activated Sepharose 4B
beads according to procedures commonly known by persons
skilled in the art. PER.C6-EPO that is secreted into the
culture medium by human EPO-producing PER.C6 cells is first
roughly separated from cell debris and other contaminants
30  by affinity column chromatography using monoclonal
antibodies specific for human EPO. Thereafter, the purified
EPO is subjected to a second chromatography procedure in

which the EPO molecules carrying GalNAc-Lewis x structures
are bound to a column containing the immobilized GalNAc-
Lewis x specific monoclonal antibodies. EPO glycoforms that
lack the GalNAc-Lewis x structures do not bind to the

5   antibodies attached to the column and are collected in the
flow-through. Bound EPO glycoforms carrying the GalNAc-
Lewis x structures are eluted from the column at low pH or
by using synthetic GalNAc-Lewis x as a competitor for
binding to the GalNAc-Lewis x specific antibodies. EPO

10  glycoforms carrying a high GalNAc-Lewis x content can be
separately eluted from the column by increasing the
concentration of GalNAc-Lewis x competitor step-wise or
gradually during the elution. EPO glycoforms with a high
GalNAc-Lewis x content are eluted at a higher concentration

15  of GalNAc-Lewis x than EPO glycoforms possessing a low
GalNAc-Lewis x content. Again, in accordance with the
methods described above, also this method enables one to
purify EPO from the culture medium by employing the
specific characteristics of the post-translational

20  modifications, such as Lewis x, lacdiNac or GalNac-Lewis x
structures brought about by the cells in which the protein
is produced. This does however, not imply that other
modifications with the (predetermined) post-translational
modifications cannot be employed for proper purification of

25  the protein.

It will be understood by those of skill in the art,
that although the invention has been illustrated with
detailed examples concerning EPO, the present invention is

30  not limited to production and/or purification of EPO with
brain-type characteristics. Various other (human)
therapeutic and/or diagnostic peptides and proteins, which

may find use in treating disorders of the brain and other parts of the central- and peripheral nervous system and/or other ischemic/reperfusion damaged tissues, can be produced by means and methods of the present invention.

5

**Example 17. EPO with a low sialic acid content has a similar potency as EPO with a high sialic acid content in reducing the infarct size after middle cerebral artery occlusion in rats.**

10        The effect of PER.C6-EPO and Eprex on the size of a brain infarct, which was experimentally induced by the occlusion of the middle cerebral artery (MCA), was studied in Wistar male rats weighing 200-250g, using a method similar to the method published by Sirén et al., 2001. The

15   right carotid artery of the animals was permanently occluded whereas the MCA was reversibly occluded for 60 min using a metal clip. Purified PER.C6-EPO with an average sialic acid content of < 6 sialic acids per molecule or Eprex (Jansen-Cilag; commercially available EPO) with an

20   average sialic acid content > 9 sialic acids per molecule) was applied intravenously at 5 min before the onset of the MCA occlusion at a dose of 5000 eU (ELISA units) per kg body weight. Notably, the sialic acid content of the PER.C6-EPO preparation ranged from 0-9 sialic acids per

25   molecule whereas Eprex contained more than 8 sialic acids per molecule. After a 60-min period, the occlusion was terminated by the removal of the metal clip surrounding the MCA. Reperfusion was observed microscopically after the removal of the clip. Twenty-four hr later the brains of the

30   living rats were examined using MRI to reveal the Apparent Diffusion Coefficient (ADC) and T2 maps. These maps were used to quantify the infarct volumes (Figs. 7A and 7B).

The results in Figs 7A and 7B show that rats treated
with the PER.C6-EPO and Eprex preparations displayed a
similar reduction in the infarct size compared to the non-
treated animals. Since the PER.C6-EPO preparation has a
much lower sialic acid content than the Eprex preparation
this result demonstrates that a high sialic content is not
essential for the neuroprotective activity of EPO in vivo.

**Example 18. Determination of half-life of EPO in rats.**
To determine the half-life of Eprex in vivo, male Wag/Rij
rats have been injected intravenously with 150 eU Eprex
diluted in PBS/0.05% Tween-80 to a final volume of 500 µl.
Just before the administration of the substrate, 200 µl of
EDTA blood was sampled as negative control using the
technique described in Lab. Animals 34, 372. At t=5, 15,
30, 60, 120, 180, 240, 300, 360, 420, 480, and 540 min
after injection 200 µl EDTA blood was taken from the
animals using the same technique. After the last blood
sampling, the animals were sacrificed. The specimen was
centrifuged at 760 x g for 15 min at RT within 30 min of
collection. The plasma samples were tested in an EPO
specific Elisa (R&D) to determine the concentration of EPO
in each sample.

As shown in Fig. 8, the decrease in the concentration
of Eprex in the plasma displays a bi-phasic curve
representing a distribution phase and a clearance phase. On
basis of these results it can be estimated that Eprex had a
half-life of about 180 min during the clearance phase. The

half-life of PER.C6-EPO is measured using the same protocol.

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**Table I.**

| Marker protein | Description |
|---|---|
| Pan-keratin | Detection of almost all cytokeratins.<br>Labels keratinized and corneal epidermis, stratified squamous epithelia of internal organs, stratified epithelia, hyperproliferative keratinocytes, and simple epithelia. |
| EMA | Epithelial membrane antigen.<br>Labels normal and neoplastic epithelium. |
| S100 | EF-band type $Ca^{2+}$ binding proteins.<br>Expressed in neural tissues and other tissues. |
| Vimentin | Cytoskeletal intermediate filaments (= structural protein).<br>Is a general marker of cells originating in the mesenchyme.<br>Expressed during skeletal muscle development. |
| Desmin | Cytoskeletal intermediate filaments (= structural protein).<br>Expressed during skeletal muscle development. |
| s.m. actin | Smooth muscle cell actin.<br>Stains smooth muscle cells and myo-epithelial cells. |
| Synaptophysin | Reacts with neuroendocrine cells. |
| Chromogranin | Acidic glycoproteins that are widely expressed within secretory granules of endocrine, neuroendocrine and neural tissue. |
| NSE | Neuron specific enolase.<br>Labels cells of neural and neuroendocrine origin |
| Neurofilament | Reacts with phosphorylated neurofilament protein and Labels neural processes and pheripheral nerves as well as symphataetic ganglion cells and adrenal medulla. |
| GFAP (polycon) | Glial Fibrillary Acidic Protein.<br>GFAP is specifically found in astroglia, which are highly responsive to neurologic insults. Astrogliosis is found to be a result of mechanical trauma, AIDS dementia and prion infection and is accompanied by an increase in GFAP expression. Immunohistochemical marker for localizing benign astrocyte and neoplastic cells of glial origin in the central nervous system. |
| CD31 | Reacts with PECAM-1.<br>Present on platelets, monocytes, granulocytes, lymphocytes, endothelial cells. |
| CD34 | Recognizes O-glycosylated transmembrane glycoprotein.<br>Expressed on hemopoietic stem cells, vascular EC, embryonic fibroblasts, some cells in fetal adult nerve tissue. |
| N-CAM | Neuronal cell adhesion molecules.<br>N-CAM is involved in cell-cell interactions during growth. |

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**Table II.**

| Marker protein | Control tissue |
|---|---|
| Pan-keratin | Colon carcinoma |
| EMA | Colon carcinoma |
| S100 | Pancreas |
| Vimentin | Tonsil |
| Desmin | Colon |
| s.m. actin | Tonsil |
| Synaptophysin | Pancreas |
| Chromogranin | Pancreas |
| NSE | Pancreas |
| Neurofilament | Colon |
| GFAP (polycon) | Brain |
| CD31 | Colon |
| CD34 | Tonsil |
| N-CAM (CD56) | Colon |

**Table III.**

| Marker protein | Supplier | Antibody | Catalog nr | Antibody dilution |
|---|---|---|---|---|
| Pan-keratin | Biogenex | Mouse IgG1 | MU071-UC | 1:200 |
| EMA | Dako | Mouse IgG2a | M0613 | 1:50 |
| S100 | Dako | Rabbit | Z0311 | 1:3000 |
| Vimentin | Biogenex | Mouse IgG1 | MU074-UC | 1:3200 |
| Desmin | Sanbio | Mouse IgG | MON 3001 | 1:50 |
| s.m. actin | Biogenex | Mouse IgG2a | MU128-UC | 1:150 |
| Synaptophysin | Dako | Mouse IgG1 | M0776 | 1:50 |
| Chromogranin | Biogenex | Mouse IgG1 | MU126-UC | 1:150 |
| NSE | Dako | Mouse IgG1 | M0873 | 1:250 |
| Neurofilament | Sanbio | Mouse IgG | MON3004 | 1:300 |
| GFAP (polycon) | Dako | Mouse IgG1 | M0761 | 1:200 |
| CD31 | Dako | Mouse IgG1 | M0823 | 1:60 |
| CD34 | Biogenex | Mouse IgG1 | MU236-UC | 1:20 |
| N-CAM (CD56) | Neomarkers | Mouse IgG1 | MS.204.P | 1:10 |

**Table IV.**

| Marker protein | Score |
|---|---|
| Pan-keratin | Negative |
| EMA | Negative |
| S100 | Negative |
| Vimentin | **Positive** |
| Desmin | Negative |
| s.m. actin | Negative |
| Synaptophysin | **Positive** |
| Chromogranin | Negative |
| NSE | Negative |
| Neurofilament | **Positive** |
| GFAP (polycon) | **Positive** |
| CD31 | Negative |
| CD34 | Negative |
| N-CAM (CD56) | **Positive** |

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**Table V.**

| Clone and culture conditions | Molar ratio of neutral monosaccharides normalized to three mannose residues | | | | |
|---|---|---|---|---|---|
| | **Man** | **Fuc** | **GalNAc** | **GlcNAc** | **Gal** |
| P8 - DMEM | 3 | 0.5 (0.9) | 0.4 (0.4) | 2.2 (2.7) | 1.7 (1.3) |
| P8 - JRH | 3 | 1.5 (1.4) | 0.7 (0.8) | 6.1 (6.4) | 3.5 (3.9) |
| P7 - DMEM | 3 | 1.5 (1.4) | 0.4 (0.3) | 5.5 (6.1) | 2.3 (3.3) |
| P7 - JRH | 3 | 1.8 (1.7) | 0.4 (0.4) | 6.1 (6.8) | 3.6 (4.2) |
| C25 - DMEM | 3 | 2.0 | 1.0 | 6.0 | 2.2 |
| Eprex | 3 | 0.7 | - | 5.4 | 4.1 |

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**Table VI.**

| P7 Mass (m/z) | Percentage of total | | | Ratio Hex:HexNAc:dHex |
|---|---|---|---|---|
| | Pool A | Pool B | Pool C | |
| **1809.64** | 2.34 | 2.99 | 2.44 | 5:4:1 |
| 1850.67 | 2.57 | 5.31 | 2.49 | 4:5:1 |
| 1891.69 | 5.06 | 10.39 | 1.31 | 3:6:1 |
| 1955.70 | - | 1.95 | 2.16 | 5:4:2 |
| 1996.72 | 6.37 | 7.96 | 6.38 | 4:5:2 |
| 2037.75 | 6.33 | 5.16 | 5.39 | 3:6:2 |
| 2053.74 | 3.70 | 4.11 | 1.98 | 5:5:1 |
| 2142.78 | 2.19 | 3.68 | 2.45 | 4:5:3 |
| **2174.77** | 6.53 | 3.63 | 8.04 | 6:5:1 |
| 2183.81 | 6.69 | 5.02 | 7.57 | 3:6:3 |
| 2199.80 | 3.78 | 4.65 | 1.58 | 4:6:2 |
| 2215.80 | 4.13 | 4.95 | 4.15 | 5:6:1 |
| 2256.82 | - | 1.30 | - | 4:7:1 |
| 2320.83 | 2.34 | 2.04 | 3.29 | 6:5:2 |
| 2361.86 | 4.35 | 3.30 | 3.23 | 5:6:2 |
| 2377.85 | 3.77 | 3.79 | 2.86 | 6:6:1 |
| 2507.91 | 1.62 | 2.32 | 1.32 | 5:6:3 |
| 2523.91 | 2.09 | 2.60 | 1.61 | 6:6:2 |
| **2539.90** | 11.89 | 4.81 | 19.32 | 7:6:1 |
| 2580.93 | 3.32 | 1.53 | 1.69 | 6:7:1 |
| 2612.94 | - | - | 1.78 | 6:5:3 |
| 2669.97 | 1.95 | 2.34 | - | 6:6:3 |
| 2685.96 | 6.21 | 3.11 | 5.81 | 7:6:2 |
| 2726.99 | 1.62 | 1.38 | 1.36 | 6:7:2 |
| 2832.02 | 3.64 | 1.55 | 3.08 | 7:6:3 |
| **2905.04** | 1.79 | - | 2.45 | 8:7:1 |
| 2978.08 | 2.23 | 1.65 | - | 7:6:4 |

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Table VII.

| P8 Mass (m/z) | Percentage of total | | | Ratio Hex:HexNAc:dHex |
|---|---|---|---|---|
| | Pool A | Pool B | Pool C | |
| **1809.64** | – | 1.03 | – | 5:4:1 |
| 1850.67 | 3.36 | 2.05 | – | 4:5:1 |
| 1891.69 | 5.11 | 2.11 | 3.04 | 3:6:1 |
| 1955.70 | 1.46 | 1.22 | 1.08 | 5:4:2 |
| 1996.72 | 5.05 | 4.61 | 6.54 | 4:5:2 |
| 2012.72 | 1.34 | 1.38 | 1.35 | 5:5:1 |
| 2037.75 | 14.62 | 14.34 | 12.48 | 3:6:2 |
| 2053.74 | 3.73 | 2.76 | 4.29 | 4:6:1 |
| 2142.78 | 2.57 | 1.97 | 2.06 | 4:5:3 |
| 2158.78 | 1.43 | 1.91 | – | 5:5:2 |
| **2174.77** | 2.40 | 2.53 | 5.58 | 6:5:1 |
| 2183.81 | 16.91 | 15.79 | 14.90 | 3:6:3 |
| 2199.80 | 1.74 | 3.18 | 4.90 | 4:6:2 |
| 2215.80 | 4.23 | 4.20 | 3.08 | 5:6:1 |
| 2256.82 | 2.08 | 3.04 | 2.17 | 4:7:1 |
| 2320.83 | 1.67 | 1.88 | 2.23 | 6:5:2 |
| 2361.86 | 3.25 | 2.25 | 3.02 | 5:6:2 |
| 2377.85 | 1.50 | 1.84 | 2.73 | 6:6:1 |
| 2402.88 | 2.05 | 2.20 | 4.26 | 4:7:2 |
| 2418.88 | 0.97 | 1.54 | – | 5:7:1 |
| 2466.89 | 1.03 | – | – | 6:5:3 |
| 2507.91 | 2.04 | 2.48 | – | 5:6:3 |
| 2523.91 | 1.58 | 1.73 | 1.47 | 6:6:2 |
| **2539.90** | 2.48 | 4.79 | 9.56 | 7:6:1 |
| 2548.94 | 1.26 | 1.14 | 0.66 | 4:7:3 |
| 2580.93 | 1.87 | 2.07 | 2.48 | 6:7:1 |
| 2685.96 | 2.74 | 3.39 | 4.30 | 7:6:2 |
| 2726.99 | 2.55 | 3.12 | – | 6:7:2 |
| 2768.01 | 1.35 | – | – | 5:8:2 |
| 2832.02 | 2.14 | 3.06 | 1.91 | 7:6:3 |
| 2873.05 | 1.70 | 1.81 | 1.63 | 6:7:3 |
| 2889.04 | 1.14 | 0.67 | – | 7:7:2 |
| 2978.08 | 0.89 | 0.99 | 2.39 | 7:6:4 |
| 3019.10 | 1.09 | 1.26 | – | 6:7:4 |

**Table VIII.**

# FT activities
(nmol/hr/mg protein)

|        | $\alpha1,2$ FT | $\alpha1,3$ FT | $\alpha1,6$ FT | GalT |
|--------|------------|------------|------------|------|
| CHO    | < 0.01     | 0.03       | 4.31       | 12.5 |
| PER.C6 | < 0.01     | 0.65       | 3.62       | 3.41 |

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CLAIMS

1. A method for identifying a mammalian cell capable of
   producing a proteinaceous molecule comprising a
   predetermined post-translational modification, said
   method comprising the steps of:
   a) analyzing the post-translational modification on
      a protein produced by said mammalian cell; and
   b) determining whether said protein comprises said
      predetermined post-translational modification.


2. A method for selecting a mammalian cell capable of
   producing a proteinaceous molecule comprising a
   predetermined post-translational modification, said
   method comprising the steps of:
   a) analyzing the presence or absence of a tissue
      specific marker or a combination of tissue
      specific markers in said mammalian cell or on the
      cell surface of said mammalian cell, which marker
      or combination of said markers is indicative for
      said predetermined post-translational
      modification to be present on said proteinaceous
      molecule; and
   b) selecting said mammalian cell on the basis of the
      presence or absence of said tissue specific
      markers.


3. A method for obtaining a mammalian cell from a
   heterogeneous cell population, said mammalian cell
   being capable of producing a proteinaceous molecule
   comprising a predetermined post-translational
   modification, said method comprising the steps of:

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a) sorting cells on the basis of the post-
   translational modifications on proteins produced
   by said cells in said heterogeneous cell
   population; and

b) selecting the cells capable of producing proteins
   comprising said predetermined post-translational
   modification.

4. A method according to any one of claims 1-3, wherein
   said predetermined post-translational modification
   comprises glycosylation.

5. A method according to claim 4, wherein said
   glycosylation comprises at least one modification
   selected from the group consisting of a Lewis x, a
   sialyl Lewis x, a GalNac structure, a GlcNac
   structure, a LacdiNAc structure, a α1,3-linked
   fucose attached to N-acetyl-glucosamine, a terminal
   N-acetyl-glucosamine, a terminal galactose, a
   bisecting N-acetyl-glucosamine, a sulphate group and
   a sialic acid.

6. A method according to any one of claims 1-5, wherein
   said mammalian cell is of neural origin.

7. A method according to any one of claims 1-6, wherein
   said mammalian cell is a human cell.

8. A method according to anyone of claims 1-7, wherein
   said mammalian cell has been immortalized.

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9. A method according to any one of claims 1-8, wherein
said mammalian cell has been provided with a nucleic
acid encoding the E1 region, or a part thereof, from
human adenovirus in such a way that said mammalian
5       cell harbors the nucleic acid in an expressible
form.

10. A method for identifying a mammalian cell capable of
producing a proteinaceous molecule comprising a
10      predetermined post-translational modification, said
method comprising the steps of:
a) providing said mammalian cell with a nucleic acid
encoding a protein capable of comprising post-
translational modifications, in such a way that
15          said mammalian cell harbors said nucleic acid in
an expressible form;
b) culturing said mammalian cell under conditions
conducive to the production of said protein;
c) analyzing the post-translational modification on
20          said protein produced by said mammalian cell; and
d) determining whether said post-translational
modification present on said protein comprises
said predetermined post-translational
modification.

25

11. A method for identifying a mammalian cell capable of
producing a proteinaceous molecule comprising a
predetermined post-translational modification, said
method comprising the steps of:
30      a) providing said mammalian cell with a nucleic acid
encoding said proteinaceous molecule capable of
comprising post-translational modifications, in

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such a way that said mammalian cell harbors said
nucleic acid in an expressible form;

b) culturing said mammalian cell under conditions
conducive to the production of said proteinaceous
molecule;

c) analyzing the post-translational modification on
said proteinaceous molecule produced by said
mammalian cell; and

d) determining whether said post-translational
modification present on said proteinaceous
molecule comprises said predetermined post-
translational modification.

12. A method according to claim 10 or 11, wherein said
protein is erythropoietin.

13. A method according to claim 10 or 11, wherein said
protein is a monoclonal antibody, or a part thereof.

14. A method according to any one of claims 10-13, wherein
said predetermined post-translational modification
comprises glycosylation.

15. A method according to claim 14, wherein said
glycosylation comprises at least one modification
selected from the group consisting of a Lewis x, a
sialyl Lewis x, a GalNac structure, a GlcNac
structure, a LacdiNAc structure, a α1,3-linked
fucose attached to N-acetyl-glucosamine, a terminal
N-acetyl-glucosamine, a terminal galactose, a
bisecting N-acetyl-glucosamine, a sulphate group and
a sialic acid.

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16. A method according to any one of claims 10-15, wherein said mammalian cell is of neural origin.

17. A method according to any one of claims 10-16, wherein said mammalian cell is a human cell.

18. A method according to anyone of claims 10-17, wherein said mammalian cell has been immortalized.

19. A method according to any one of claims 10-16, wherein said mammalian cell has been provided with a nucleic acid encoding the E1 region, or a part thereof, from human adenovirus in such a way that said mammalian cell harbors the nucleic acid in an expressible form.

20. A method for producing a proteinaceous molecule comprising a predetermined post-translational modification, comprising the steps of:

   a) providing a mammalian cell obtainable by a method according to any one of claims 1-9, with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form; and

   b) culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule,

   wherein said proteinaceous molecule is not human erythropoietin or a fully human monoclonal antibody.

21. A method according to claim 20, wherein said brain-
    type modification comprises glycosylation.

22. A method according to claim 21, wherein said
    glycosylation comprises at least one modification
    selected from the group consisting of a Lewis x, a
    sialyl Lewis x, a GalNac structure, a GlcNac
    structure, a LacdiNAc structure, a α1,3-linked
    fucose attached to N-acetyl-glucosamine, a terminal
    N-acetyl-glucosamine, a terminal galactose, a
    bisecting N-acetyl-glucosamine, a sulphate group and
    a sialic acid.

23. A method for producing a proteinaceous molecule
    comprising a predetermined post-translational
    modification, comprising the steps of:
    a) identifying a mammalian cell having the ability
       to provide said proteinaceous molecule with said
       predetermined post-translational modification;
    b) providing said mammalian cell with a nucleic acid
       encoding said proteinaceous molecule in such a
       way that said mammalian cell harbors said nucleic
       acid in an expressible form; and
    c) culturing said mammalian cell under conditions
       conducive to the production of said proteinaceous
       molecule.

24. A method for producing a proteinaceous molecule
    comprising a predetermined post-translational
    modification, said method comprising the steps of:

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    a) identifying a mammalian cell having the ability
       to provide said proteinaceous molecule with said
       predetermined post-translational modification;

    b) providing said mammalian cell with a nucleic acid
       encoding said proteinaceous molecule in such a
       way that said mammalian cell harbors said nucleic
       acid in an expressible form;

    c) culturing said mammalian cell under conditions
       conducive to the production of said proteinaceous
       molecule;

    d) analyzing said post-translational modifications
       on said proteinaceous molecule so produced; and

    e) determining whether said post-translational
       modification present on said proteinaceous
       molecule comprises said predetermined post-
       translational modification.

25. A method according to claim 23 or 24, comprising the
    extra step of purifying said proteinaceous molecule
    from the mammalian cell culture.

26. A method according to any of claims 23-25, wherein
    said proteinaceous molecule is erythropoietin.

27. A method according to any of claims 23-25, wherein
    said proteinaceous molecule is a monoclonal
    antibody, or a part thereof.

28. A method according to any one of claims 23-27, wherein
    said predetermined post-translational modification
    comprises glycosylation.

29. A method according to claim 28, wherein said
    glycosylation comprises at least one modification
    selected from the group consisting of a Lewis x, a
    sialyl Lewis x, a GalNac structure, a GlcNac
5   structure, a LacdiNAc structure, a α1,3-linked
    fucose attached to N-acetyl-glucosamine, a terminal
    N-acetyl-glucosamine, a terminal galactose, a
    bisecting N-acetyl-glucosamine, a sulphate group and
    a sialic acid.

10

30. A method according to any one of claims 23-29, wherein
    said mammalian cell is of neural origin.

31. A method according to any one of claims 23-30, wherein
15      said mammalian cell is a human cell.

32. A method according to anyone of claims 23-31, wherein
    said mammalian cell has been immortalized prior or
    subsequent to step a).

20

33. A method according to any one of claims 23-32, wherein
    said mammalian cell has been provided with a nucleic
    acid encoding the E1 region, or a part thereof, from
    human adenovirus in such a way that said mammalian
25      cell harbors the nucleic acid in an expressible
    form.

34. A method according to claim 33, wherein said mammalian
    cell is PER.C6, deposited under No. 96022940 at the
30      European Collection of Animal Cell Cultures at the
    Center for Applied Microbiology and Research.

35. A method for producing a proteinaceous molecule
    comprising a predetermined post-translational
    modification, said method comprising the steps of:
    a) providing a mammalian cell obtainable by a method
       according to any one of claims 1-9, with a
       nucleic acid encoding said proteinaceous molecule
       in such a way that said mammalian cell harbors
       said nucleic acid in an expressible form;
    b) culturing said mammalian cell under conditions
       conducive to the production of said proteinaceous
       molecule, and
    c) purifying said proteinaceous molecule from the
       mammalian cell culture.

36. A method for producing a proteinaceous molecule
    comprising a predetermined post-translational
    modification, said method comprising the steps of:
    a) providing a mammalian cell obtainable by a method
       according to any one of claims 1-9, with a
       nucleic acid encoding said proteinaceous molecule
       in such a way that said mammalian cell harbors
       said nucleic acid in an expressible form;
    b) culturing said mammalian cell under conditions
       conducive to the production of said proteinaceous
       molecule;
    c) analyzing said post-translational modifications
       on said proteinaceous molecule so produced; and
    d) determining whether said post-translational
       modification present on said proteinaceous
       molecule comprises said predetermined post-
       translational modification.

37. A method according to claim 36, comprising the extra step of purifying said proteinaceous molecule from the mammalian cell culture.

38. A method according to any one of claims 35-37, wherein said proteinaceous molecule is erythropoietin.

39. A method according to any one of claims 35-37, wherein said proteinaceous molecule is a monoclonal antibody, or a part thereof.

40. A method according to any one of claims 35-39, wherein said predetermined post-translational modification comprises glycosylation.

41. A method according to claim 40, wherein said glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a $\alpha$1,3-linked fucose attached to N-acetyl-glucosamine, a terminal N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

42. A method according to claim 25, wherein said purification comprises a step that employs said predetermined post-translational modification.

43. A method according to claim 42, wherein said proteinaceous molecule is erythropoietin.

44. A method according to claim 42, wherein said proteinaceous molecule is monoclonal antibody, or a part thereof.

5      45. A method according to any one of claims 42-44, wherein said predetermined post-translational modification comprises glycosylation.

46. A method according to claim 45, wherein said
10         glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a terminal
15         N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

47. A method according to any one of claims 42-46, wherein
20         said mammalian cell is of neural origin.

48. A method according to any one of claims 42-47, wherein said mammalian cell is a human cell.

25     49. A method according to any one of claims 42-48, wherein said mammalian cell has been immortalized prior or subsequent to step a).

50. A method according to any one of claims 42-49, wherein
30         said mammalian cell has been provided with a nucleic acid encoding the E1 region, or a part thereof, from human adenovirus in such a way that said mammalian

cell harbors the nucleic acid in an expressible form.

51. A method according to claim 50, wherein said mammalian cell is PER.C6, deposited under No. 96022940 at the European Collection of Animal Cell Cultures at the Center for Applied Microbiology and Research.

52. A method according to claim 35 or 37, wherein said purification comprises a step that employs said predetermined post-translational modification.

53. A method according to claim 52, wherein said predetermined post-translational modification comprises glycosylation.

54. A method according to claim 53, wherein said glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a terminal N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

55. A method according to any one of claims 42-54, wherein said purification comprises a step in which an antibody is employed that is specific for an epitope present in said predetermined post-translational modification.

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56. A method according to any one of claims 42-55, wherein said proteinaceous molecule is purified to homogeneity.

5      57. A pharmaceutically acceptable composition comprising a proteinaceous molecule having a predetermined post-translational modification, obtainable according to any one of claims 20-56, and a pharmaceutically acceptable carrier.

10

58. A pharmaceutically acceptable composition according to claim 57, wherein said proteinaceous molecule is erythropoietin.

15     59. A pharmaceutically acceptable composition according to claim 58, wherein said erythropoietin has a lower erythropoietic effect as compared to erythropoietin not having said predetermined post-translational modification.

20

60. Recombinantly produced erythropoietin comprising at least one post-translational modification selected from the group consisting of: a sialyl Lewis x structure, a Lewis x structure, a $\alpha 1,3$-linked fucose

25     attached to N-acetyl-glucosamine, a LacdiNAc structure, a terminal N-acetyl-glucosamine group and a terminal galactose group.

61. Recombinantly produced erythropoietin according to

30     claim 60, wherein said erythropoietin is produced on a mammalian cell obtainable according to any one of claims 1-11.

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62. Use of PER.C6 for the production of a proteinaceous
    molecule comprising a predetermined post-
    translational modification.

5

63. Use of PER.C6 according to claim 62, wherein said
    proteinaceous molecule is erythropoietin.

64. Use of PER.C6 according to claim 62, wherein said
10      proteinaceous molecule is a monoclonal antibody, or
        a part thereof.

65. A proteinaceous molecule obtainable according to any
        one of claims 20-56 for the treatment of a human or
15      a human body by surgery, therapy or diagnosis.

66. Use of a proteinaceous molecule obtainable according
        to any one of claims 20-56 for the manufacture of a
        medicament for the treatment of hypoxia-induced
20      disorders, neurodegenerative afflictions, or acute
        damage to the central- or peripheral nervous system.

67. Use of a proteinaceous molecule obtainable according
        to anyone of claims 20-56 for the manufacture of a
25      medicament for the treatment of ischemia/reperfusion
        injuries.

68. Use of recombinantly produced erythropoietin according
        to claim 60 or 61 for the preparation of a
30      medicament for the treatment of hypoxia-induced
        disorders, neurodegenerative afflictions, or acute
        damage to the central- or peripheral nervous system.

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69. Use of recombinantly produced erythropoietin
    according to claim 60 or 61 for the preparation of a
    medicament for the treatment of ischemia/reperfusion
5   injuries.

70. Use of recombinantly produced erythropoietin
    according to claim 60 or 61 for the preparation of a
    medicament for the treatment of inflammatory
10  disease.

71. Use of recombinantly produced erythropoietin
    according to claim 60 or 61 for the preparation of a
    medicament for the treatment of an immune disorder.

15

72. Use of a composition of erythropoietin-like molecules
    selected from the group consisting of one or more
    muteins of erythropoietin, one or more derivatives
    of erythropoietin, and a composition of one or more
20  fractions of erythropoietin molecules sialylated to
    a varying degree, for the preparation of a
    medicament for the treatment of a disorder selected
    from the group consisting of ischemia, a reperfusion
    injury, a hypoxia-induced disorder, an inflammatory
25  disease, a neurodegenerative disorder, and acute
    damage to the central- or peripheral nervous system,
    wherein said composition of erythropoietin-like
    molecules has on a protein content basis a lower
    erythropoietic activity *in vivo* than epoetin alfa.

30

73. Use of a composition of erythropoietin-like molecules
    selected from the group consisting of one or more

muteins of erythropoietin, one or more derivatives
of erythropoietin, and a composition of one or more
fractions of erythropoietin molecules sialylated to
a varying degree, for the preparation of a
5     medicament for the treatment of a disorder selected
from the group consisting of ischemia, a reperfusion
injury, a hypoxia-induced disorder, an inflammatory
disease, a neurodegenerative disorder or acute
damage to the central or peripheral nervous system,
10    wherein said composition of erythropoietin-like
molecules is characterized by an average number of
sialic acid residues that is at least 10% lower than
the average number of sialic acid residues per
erythropoietin molecule in epoetin alfa.

15

74.   Use according to claim 73, wherein the average number
of sialic acid residues per erythropoietin-like
molecule is at least 20% lower than the average
number of sialic acid residues per molecule in
20    epoetin alfa.

75.   Use according to claim 73, wherein the average number
of sialic acid residues per erythropoietin-like
molecule is at least 30% lower than the average
25    number of sialic acid residues per molecule in
epoetin alfa.

76.   Use according to claim 73, wherein the average number
of sialic acid residues per erythropoietin-like
30    molecule is at least 40% lower than the average
number of sialic acid residues per molecule in

epoetin alfa.

77.  Use according to claim 73, wherein the average number
     of sialic acid residues per erythropoietin-like
     molecule is at least 50% lower than the average
     number of sialic acid residues per molecule in
     epoetin alfa.

78.  Use according to claim 73, wherein the average number
     of sialic acid residues per erythropoietin-like
     molecule is at least 60% lower than the average
     number of sialic acid residues per molecule in
     epoetin alfa.

79.  Use according to claim 73, wherein the average number
     of sialic acid residues per erythropoietin-like
     molecule is at least 70% lower than the average
     number of sialic acid residues per molecule in
     epoetin alfa.

80.  Use according to claim 73, wherein the average number
     of sialic acid residues per erythropoietin-like
     molecule is at least 80% lower than the average
     number of sialic acid residues per molecule in
     epoetin alfa.

81.  Use according to claim 73, wherein the average number
     of sialic acid residues per erythropoietin-like
     molecule is at least 90% lower than the average
     number of sialic acid residues per molecule in
     epoetin alfa.

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82. Use according to any one of claims 73-81, wherein
    said composition of erythropoietin-like molecules
    has been obtained by subjecting erythropoietin or an
    erythropoietin-like molecule to treatment to lower
    the average number of sialic acid residues per
    erythropoietin-like molecule.

83. Use according to claim 82, wherein said treatment
    comprises the step of subjecting the erythropoietin
    or erythropoietin-like molecule to the action of an
    enzyme or chemical capable of splitting a glycosidic
    bond.

84. Use according to any one of claims 73-81, wherein
    said composition of erythropoietin-like molecules
    has been obtained by separation from fractions
    comprising erythropoietin-like molecules that have a
    higher sialic acid content.

85. Use according to claim 72 to 83, wherein said
    composition of erythropoietin-like molecules is
    obtainable by recombinant expression of
    erythropoietin or a mutein of erythropoietin in
    transfected Chinese Hamster Ovary cells, Baby
    Hamster Kidney cells, or transfected human cells
    followed by one or more purification steps.

86. Use according to any one of claims 73-81, wherein
    said erythropoietin-like molecules have been
    produced by a method according to any one of claims
    23-26.

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87. Use according to any one of claims 73-81, wherein
    said erythropoietin-like molecules have been
    produced on an immortalized human cell.

88. Use of a composition of erythropoietin-like molecules
    selected from the group consisting of one or more
    muteins of erythropoietin, one or more derivatives
    of erythropoietin, and a composition of one or more
    fractions of erythropoietin molecules sialylated to
    a varying degree, for the preparation of a
    medicament for the treatment of a disorder selected
    from the group consisting of ischemia, a reperfusion
    injury, a hypoxia-induced disorder, an inflammatory
    disease, a neurodegenerative disorder or acute
    damage to the central or peripheral nervous system,
    wherein said composition is characterized by the
    presence of erythropoietin-like molecules that once
    administered parenterally to a human or an animal
    subject are cleared from the bloodstream at a faster
    rate than epoetin alfa.

89. A pharmaceutical preparation comprising
    erythropoietin-like molecules selected from the
    group consisting of one or more muteins of
    erythropoietin, one or more derivatives of
    erythropoietin, and a composition of one or more
    fractions of erythropoietin or erythropoietin-like
    molecules sialylated to a varying degree, said
    preparation being characterized in having a lower
    erythropoietic activity in vivo on a protein content
    basis than epoetin alfa.

90. A pharmaceutical preparation comprising
erythropoietin-like molecules selected from the
group consisting of one or more muteins of
erythropoietin, one or more derivatives of
erythropoietin, and a composition of one or more
fractions of erythropoietin or erythropoietin-like
molecules sialylated to a varying degree, wherein
said erythropoietin-like molecules are characterized
by an average number of sialic acid residues that is
at least 10% lower than the average number of sialic
acid residues per erythropoietin molecule in epoetin
alfa.

91. A pharmaceutical preparation according to claim 90,
wherein said erythropoietin-like molecules are
characterized by an average number of sialic acid
residues that is at least 20% lower than the average
number of sialic acid residues per erythropoietin
molecule in epoetin alfa.

92. A pharmaceutical preparation according to claim 90,
wherein said erythropoietin-like molecules are
characterized by an average number of sialic acid
residues that is at least 30% lower than the average
number of sialic acid residues per erythropoietin
molecule in epoetin alfa.

93. A pharmaceutical preparation according to claim 90,
wherein said erythropoietin-like molecules are
characterized by an average number of sialic acid
residues that is at least 40% lower than the average
number of sialic acid residues per erythropoietin

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molecule in epoetin alfa.

94. A pharmaceutical preparation according to claim 90,
    wherein said erythropoietin-like molecules are
    characterized by an average number of sialic acid
    residues that is at least 50% lower than the average
    number of sialic acid residues per erythropoietin
    molecule in epoetin alfa.

95. A pharmaceutical preparation according to claim 90,
    wherein said erythropoietin-like molecules are
    characterized by an average number of sialic acid
    residues that is at least 60% lower than the average
    number of sialic acid residues per erythropoietin
    molecule in epoetin alfa.

96. A pharmaceutical preparation according to claim 90,
    wherein said erythropoietin-like molecules are
    characterized by an average number of sialic acid
    residues that is at least 70% lower than the average
    number of sialic acid residues per erythropoietin
    molecule in epoetin alfa.

97. A pharmaceutical preparation according to claim 90,
    wherein said erythropoietin-like molecules are
    characterized by an average number of sialic acid
    residues that is at least 80% lower than the average
    number of sialic acid residues per erythropoietin
    molecule in epoetin alfa.

98. A pharmaceutical preparation according to claim 90,
    wherein said erythropoietin-like molecules are

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characterized by an average number of sialic acid residues that is at least 90% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

99. A pharmaceutical preparation according to any one of claims 90-98, wherein said erythropoietin-like molecules have been obtained by subjecting erythropoietin-like molecules to treatment to lower the average number of sialic acid residues per erythropoietin-like molecule.

100. A pharmaceutical preparation according to claim 99, wherein said treatment comprises the step of subjecting the erythropoietin-like molecules to an enzyme or a chemical capable of splitting a glycosidic bond.

101. A pharmaceutical preparation according to any one of claims 90-98, wherein said erythropoietin-like molecules have been obtained by separation from fractions comprising erythropoietin-like molecules that have a higher sialic acid content.

102. A pharmaceutical preparation according to any one of claims 90-101, wherein said erythropoietin-like molecules are obtainable by recombinant expression of erythropoietin or a mutein of erythropoietin in transfected Chinese Hamster Ovary cells, Baby Hamster Kidney cells, or transfected human cells followed by one or more purification steps.

103. A pharmaceutical preparation according to any one of
claims 90-98, wherein said erythropoietin-like
molecules have been produced by a method according
to any one of claims 23-26.

104. A pharmaceutical preparation comprising
erythropoietin-like molecules selected from the
group consisting of one or more muteins of
erythropoietin, one or more derivatives of
erythropoietin, and a composition of one or more
fractions of erythropoietin or erythropoietin-like
molecules sialylated to a varying degree, said
preparation being characterized by the presence of
erythropoietin-like molecules that once administered
parenterally to a human or an animal subject are
cleared from the bloodstream at a faster rate than
epoetin alfa.

105. A method for the preventative and/or therapeutic
treatment of a disorder selected from the group
consisting of ischemia, a reperfusion injury, a
hypoxia-induced disorder, an inflammatory disease, a
neurodegenerative disorder, and acute damage to the
central- or peripheral nervous system, said method
comprising the step of administering to a human or
animal subject a composition of erythropoietin-like
molecules selected from the group consisting of one
or more muteins of erythropoietin, one or more
derivatives of erythropoietin, and a composition of
one or more fractions of erythropoietin or
erythropoietin-like molecules sialylated to a
varying degree, wherein said composition is

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characterized by the presence of erythropoietin-like
molecules that once administered parenterally to a
human or an animal subject are cleared from the
bloodstream at a faster rate than epoetin alfa.

106. A method for the preventative and/or therapeutic
treatment of a disorder selected from the group
consisting of ischemia, a reperfusion injury, a
hypoxia-induced disorder, an inflammatory disease, a
neurodegenerative disorder, and acute damage to the
central- or peripheral nervous system, said method
comprising the step of administering to a human or
animal subject a composition of erythropoietin-like
molecules selected from the group consisting of one
or more muteins of erythropoietin, one or more
derivatives of erythropoietin, and a composition of
one or more fractions of erythropoietin or
erythropoietin-like molecules sialylated to a
varying degree, wherein said composition of
erythropoietin-like molecules is characterized by an
average number of sialic acid residues that is at
least 10% lower than the average number of sialic
acid residues per erythropoietin molecule in epoetin
alfa.

107. A method according to claim 106, wherein the average
number of sialic acid residues per erythropoietin-
like molecule is at least 20% lower than the average
number of sialic acid residues per molecule in
epoetin alfa.

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108. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 30% lower than the average number of sialic acid residues per molecule in epoetin alfa.

109. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 40% lower than the average number of sialic acid residues per molecule in epoetin alfa.

110. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 50% lower than the average number of sialic acid residues per molecule in epoetin alfa.

111. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 60% lower than the average number of sialic acid residues per molecule in epoetin alfa.

112. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 70% lower than the average number of sialic acid residues per molecule in epoetin alfa.

113. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-

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like molecule is at least 80% lower than the average
number of sialic acid residues per molecule in
epoetin alfa.

5      114. A method according to claim 106, wherein the average
number of sialic acid residues per erythropoietin-
like molecule is at least 90% lower than the average
number of sialic acid residues per molecule in
epoetin alfa.

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115. A method according to any one of claims 106-114,
wherein said erythropoietin-like molecules have been
obtained by subjecting erythropoietin-like molecules
to treatment to lower the average number of sialic

15        acid residues per erythropoietin-like molecule.

116. A method according to claim 115, wherein said
treatment comprises the step of subjecting
erythropoietin-like molecules to the action of an

20        enzyme or a chemical capable of splitting a
glycosidic bond.

117. A method according to any one of claims 106-116,
wherein said erythropoietin-like molecules have been

25        obtained by separation from fractions comprising
erythropoietin-like molecules that have a higher
sialic acid content.

118. A method according to any one of claims 105-117,

30        wherein said erythropoietin-like molecules are
obtainable by recombinant expression of
erythropoietin or a mutein of erythropoietin in

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transfected Chinese Hamster Ovary cells, Baby
Hamster Kidney cells, or transfected human cells
followed by one or more purification steps.

5      119. A method according to any one of claims 106-114,
      wherein said erythropoietin-like molecules have been
      produced by a method according to any one of claims
      23-26.

10     120. A method for treatment or preventing a disorder
      selected from the group consisting of ischemia, a
      reperfusion injury, a hypoxia-induced disorder, an
      inflammatory disease, a neurodegenerative disorder,
      and acute damage to the central- or peripheral
15      nervous system, said method comprising the step of
      administering to a human or animal subject a
      composition of erythropoietin-like molecules
      selected from the group consisting of one or more
      muteins of erythropoietin, one or more derivatives
20      of erythropoietin, or a composition of one or more
      fractions of erythropoietin molecules sialylated to
      a varying degree, wherein said composition is
      characterized by the presence of erythropoietin-like
      molecules that once administered parenterally to a
25      human or an animal subject are cleared from the
      bloodstream at a faster rate than epoetin alfa.

      121. A composition of erythropoietin-like molecules
      selected from the group consisting of one or more
30      muteins of erythropoietin, one or more derivatives
      of erythropoietin, and a composition of one or more
      fractions of erythropoietin or erythropoietin-like

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molecules sialylated to a varying degree, for the
preventative and/or therapeutic treatment of the
human or animal body, wherein said composition of
erythropoietin-like molecules has on a protein

5       content basis a lower erythropoietic activity *in
vivo* than epoetin alfa.


122. A composition of erythropoietin-like molecules
selected from the group consisting of one or more

10      muteins of erythropoietin, one or more derivatives
of erythropoietin, and a composition of one or more
fractions of erythropoietin or erythropoietin-like
molecules sialylated to a varying degree, for the
preventative and/or therapeutic treatment of the

15      human or animal body, wherein said composition of
erythropoietin-like molecules is characterized by an
average number of sialic acid residues that is at
least 10% lower than the average number of sialic
acid residues per erythropoietin molecule in epoetin

20      alfa.


123. A composition according to claim 122 for the
preventative and/or therapeutic treatment of the
human or animal body, wherein the average number of

25      sialic acid residues per erythropoietin-like
molecule is at least 20% lower than the average
number of sialic acid residues per molecule in
epoetin alfa.


30  124. A composition according to claim 122 for the
preventative and/or therapeutic treatment of the
human or animal body, wherein the average number of

sialic acid residues per erythropoietin-like
molecule is at least 30% lower than the average
number of sialic acid residues per molecule in
epoetin alfa.

125. A composition according to claim 122 for the
preventative and/or therapeutic treatment of the
human or animal body, wherein the average number of
sialic acid residues per erythropoietin-like
molecule is at least 40% lower than the average
number of sialic acid residues per molecule in
epoetin alfa.

126. A composition according to claim 122 for the
preventative and/or therapeutic treatment of the
human or animal body, wherein the average number of
sialic acid residues per erythropoietin-like
molecule is at least 50% lower than the average
number of sialic acid residues per molecule in
epoetin alfa.

127. A composition according to claim 122 for the
preventative and/or therapeutic treatment of the
human or animal body, wherein the average number of
sialic acid residues per erythropoietin-like
molecule is at least 60% lower than the average
number of sialic acid residues per molecule in
epoetin alfa.

128. A composition according to claim 122 for the
preventative and/or therapeutic treatment of the
human or animal body, wherein the average number of

sialic acid residues per erythropoietin-like molecule is at least 70% lower than the average number of sialic acid residues per molecule in epoetin alfa.

129. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 80% lower than the average number of sialic acid residues per molecule in epoetin alfa.

130. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 90% lower than the average number of sialic acid residues per molecule in epoetin alfa.

131. A composition according to any one of claims 122-130 for the preventative and/or therapeutic treatment of the human or animal body, wherein said composition of erythropoietin-like molecules has been obtained by subjecting erythropoietin-like molecules to treatment with an enzyme such as neuraminidase or a chemical substance such as an acid to lower the average number of sialic acid residues per erythropoietin-like molecule.

132. A composition according to any one of claims 122-130 for the preventative and/or therapeutic treatment of the human or animal body, wherein said composition of erythropoietin-like molecules has been obtained by separation from fractions comprising erythropoietin-like molecules that have a higher sialic acid content.

133. A composition according to any one of claim 121-132 for the preventative and/or therapeutic treatment of the human or animal body, wherein said erythropoietin-like molecules are obtainable by recombinant expression of erythropoietin or a mutein of erythropoietin in transfected Chinese Hamster Ovary cells, Baby Hamster Kidney cells, or transfected human cells followed by one or more purification steps.

134. A composition according to any one of claims 122-130 for the preventative and/or therapeutic treatment of the human or animal body, wherein said erythropoietin-like molecules have been produced by a method according to any one of claims 23-26 for the preventative and/or therapeutic treatment of the human or animal body.

135. A composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin or erythropoietin-like molecules sialylated to a varying degree, for the

preventative and/or therapeutic treatment of the human or animal body, wherein said composition of erythropoietin-like molecules is characterized by the presence of erythropoietin-like molecules that once administered parenterally to a human or an animal subject are cleared from the bloodstream at a faster rate than epoetin alfa.
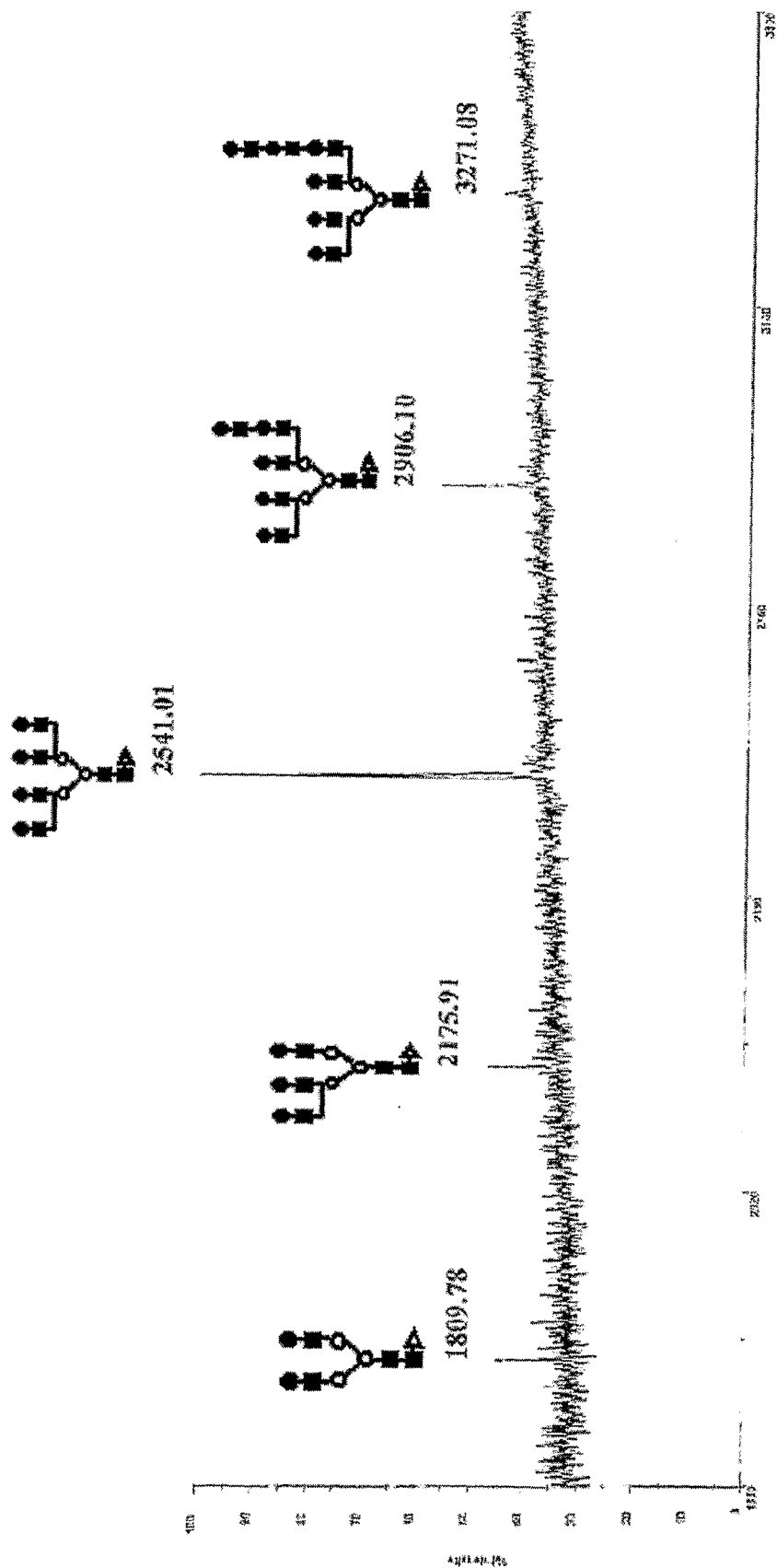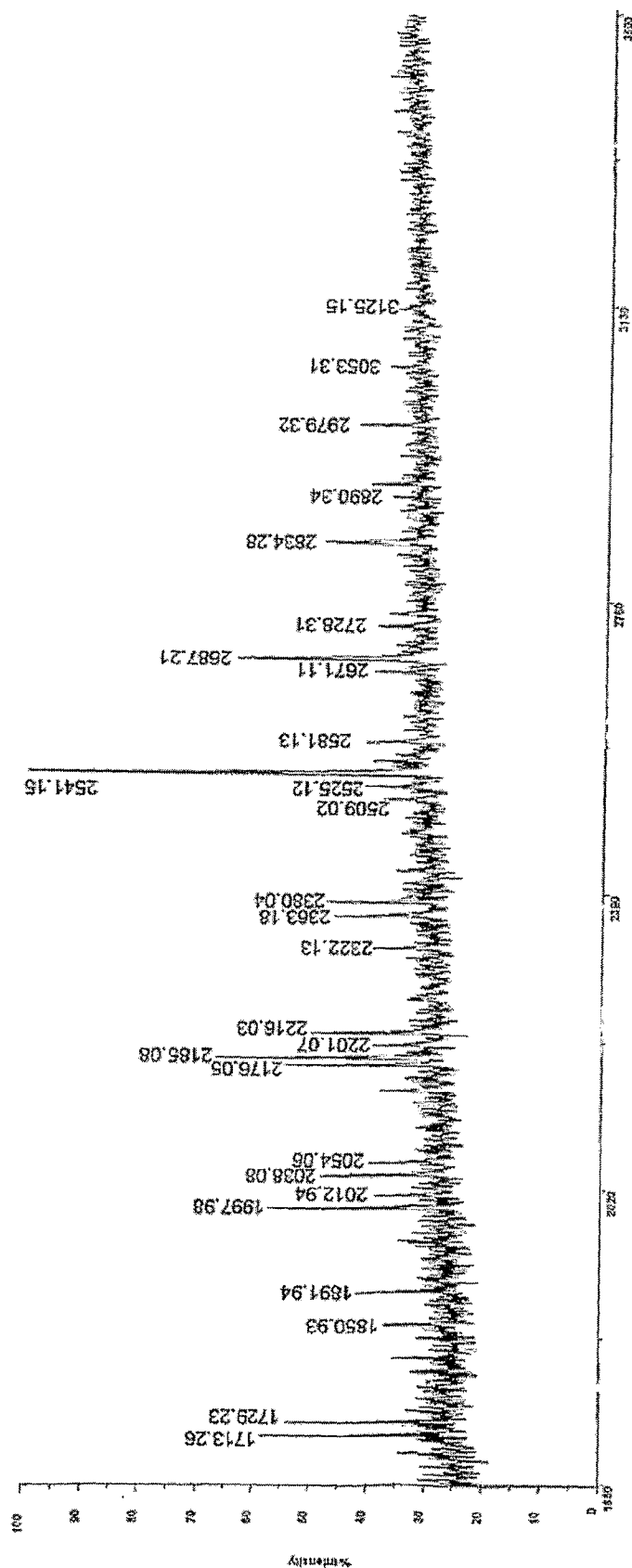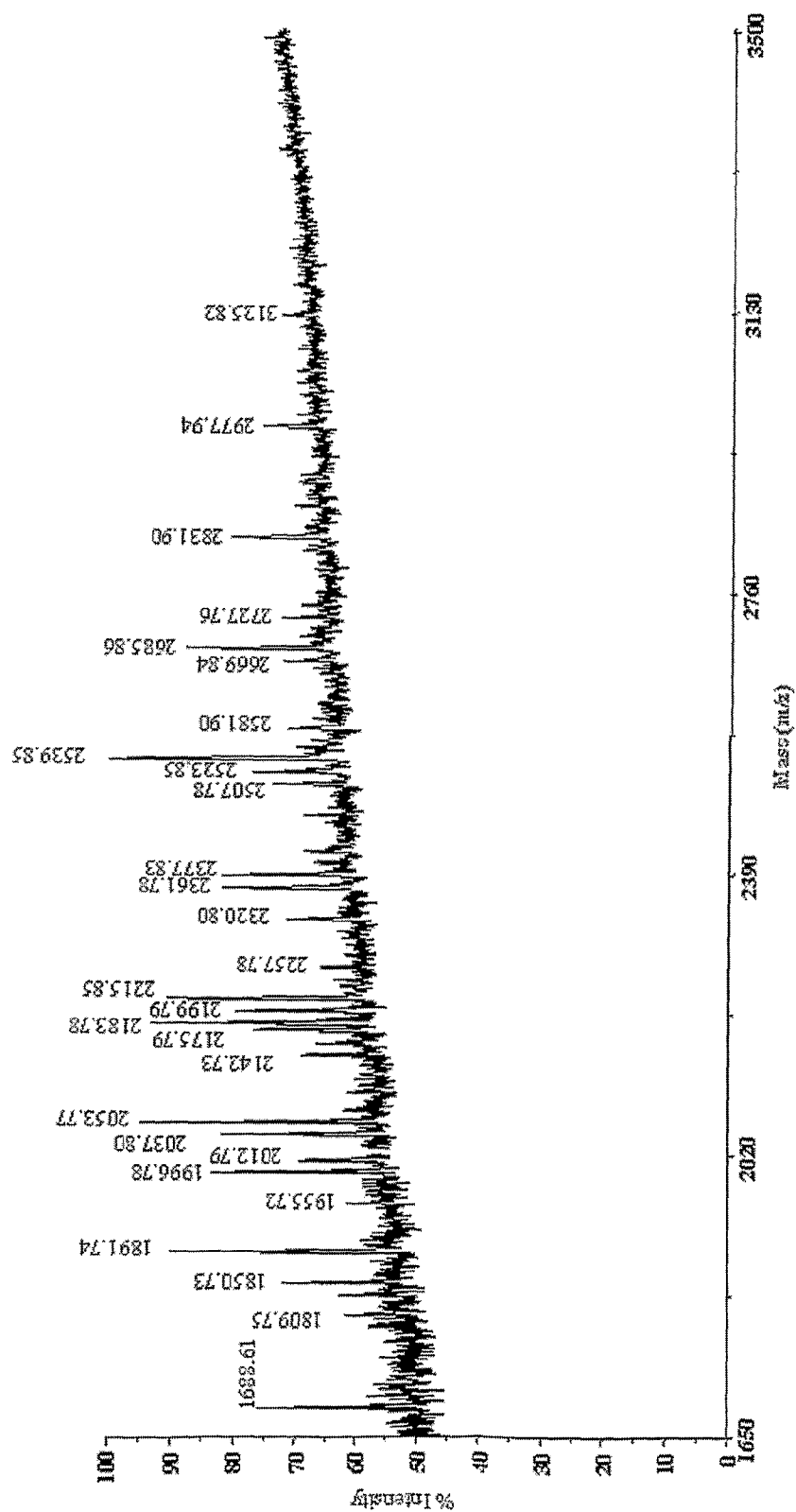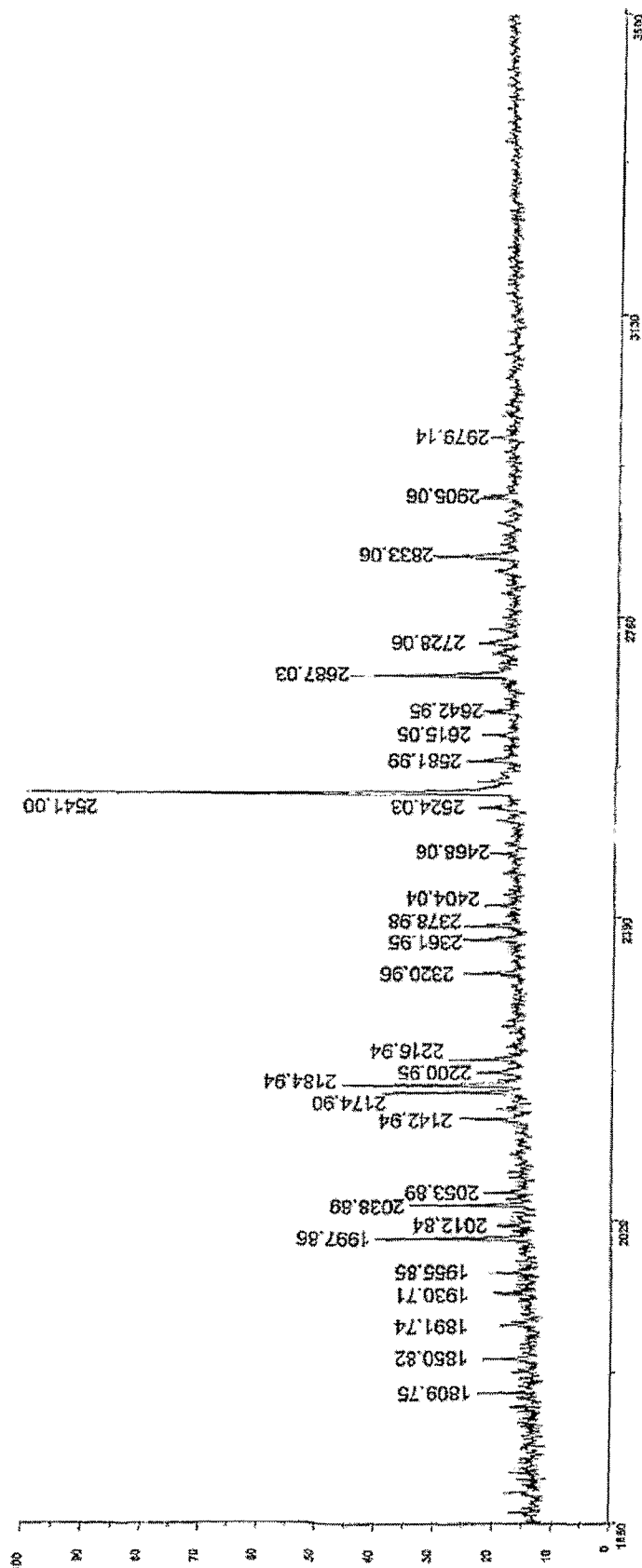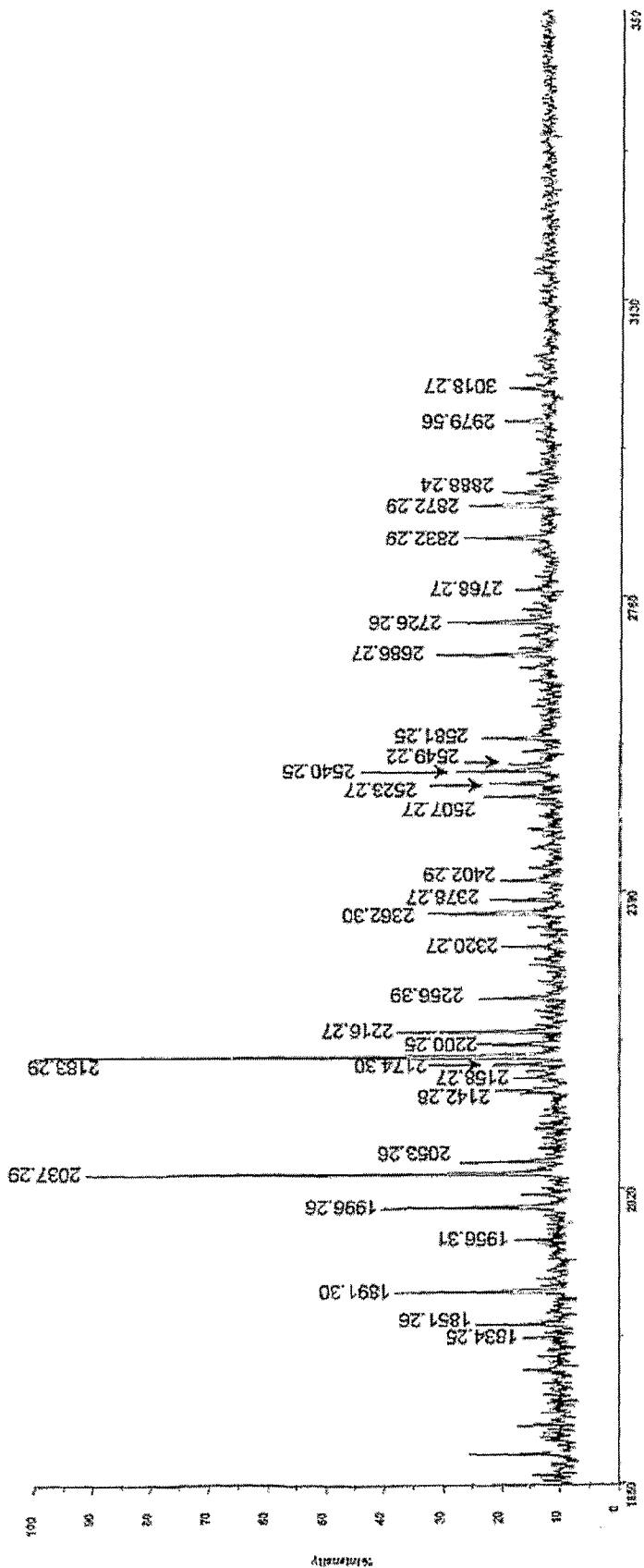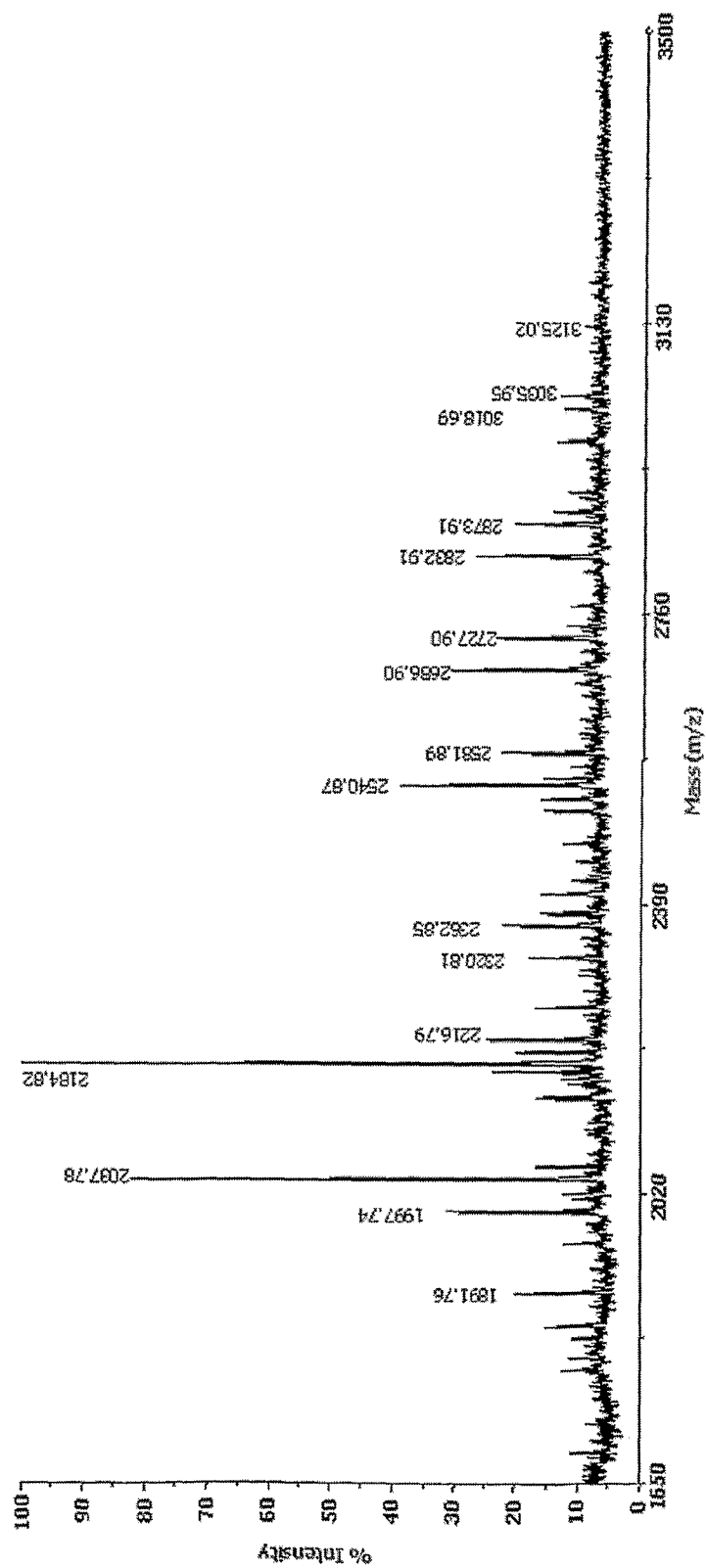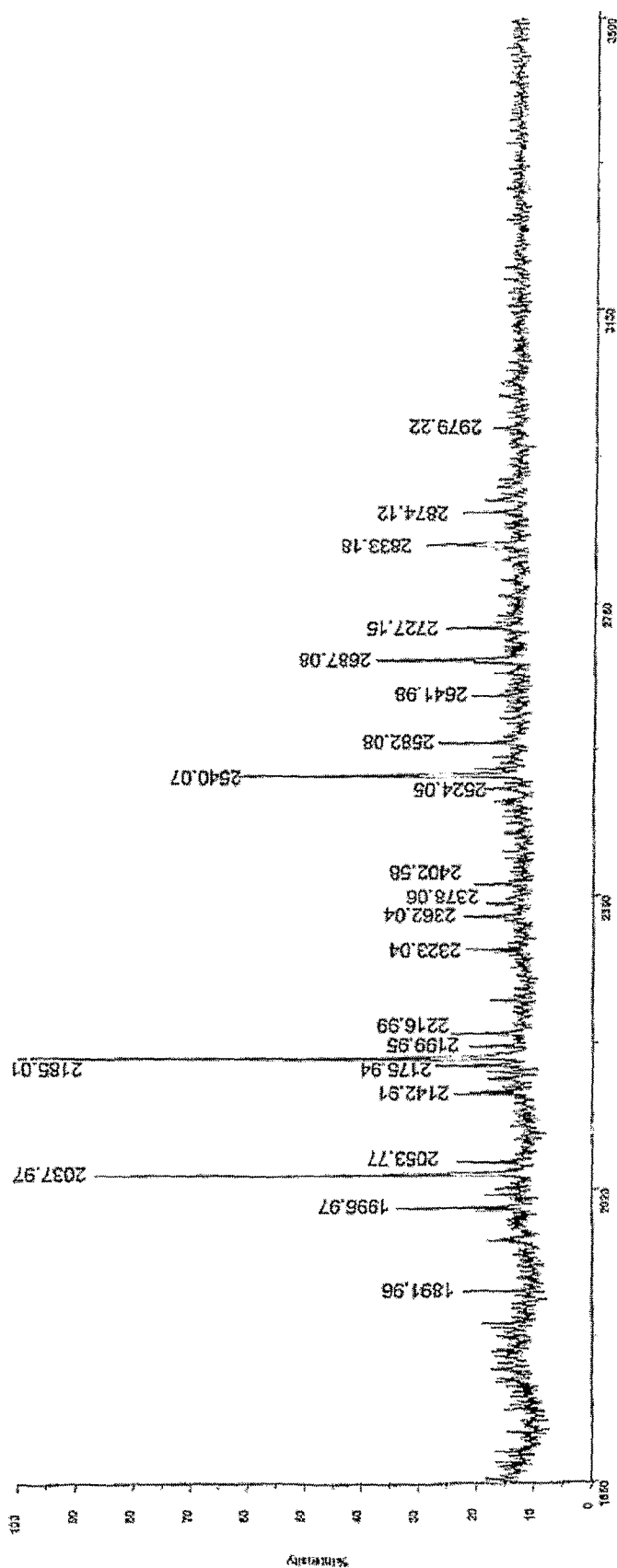
Fig. 1A

Fig. 1B

Fig. 1C

Fig. 1D

Fig. 1E

Fig. 1F

Fig. 1G

Fig. 2

Fig. 3

Fig. 4



CHO          PER.C6

mAb α Le^x

mAb α sialyl Le^x

Fig. 5



Lewis x　　　　Sialyl-Lewis x
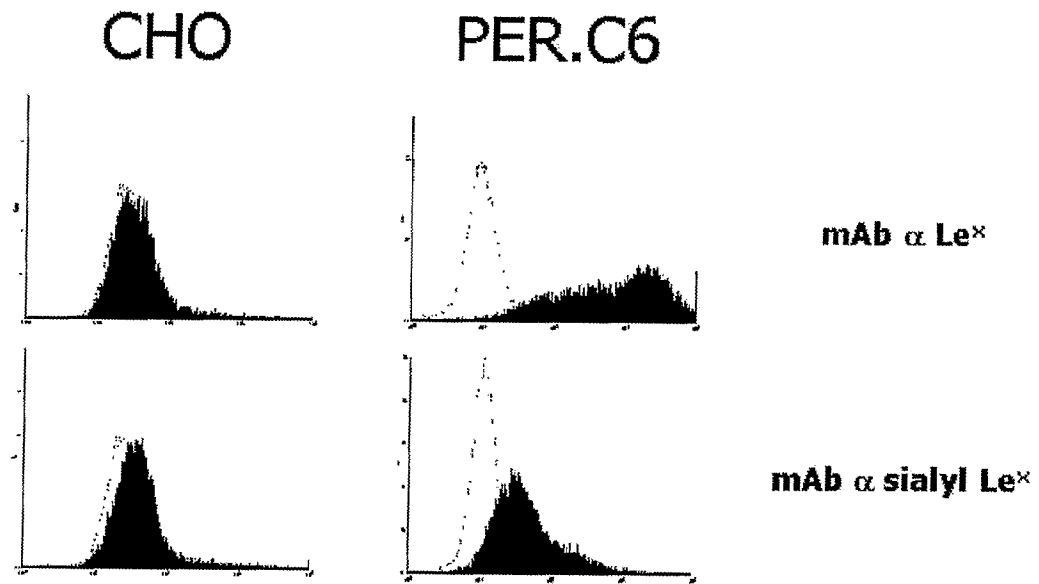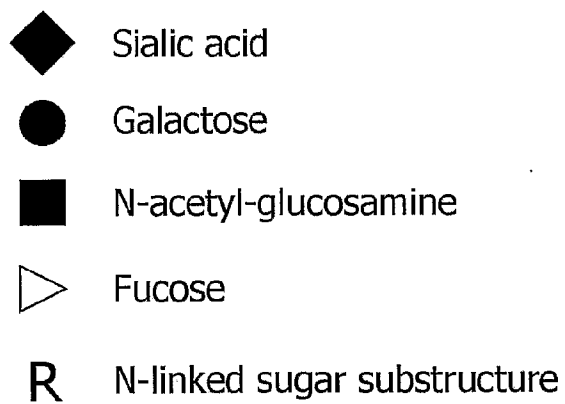
◆ Sialic acid

● Galactose

■ N-acetyl-glucosamine

▷ Fucose

R N-linked sugar substructure
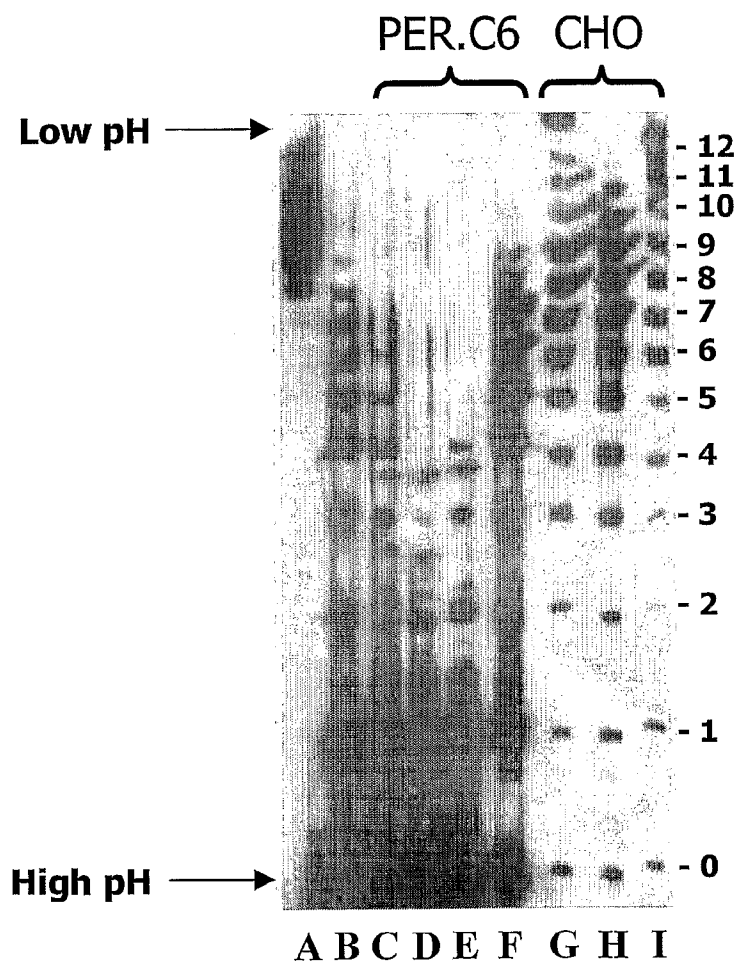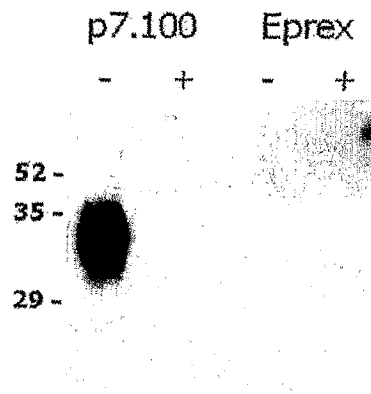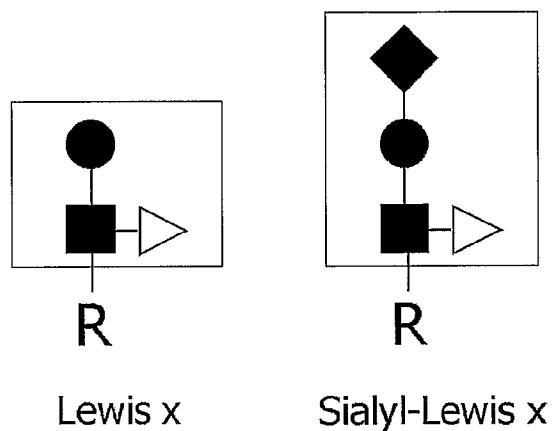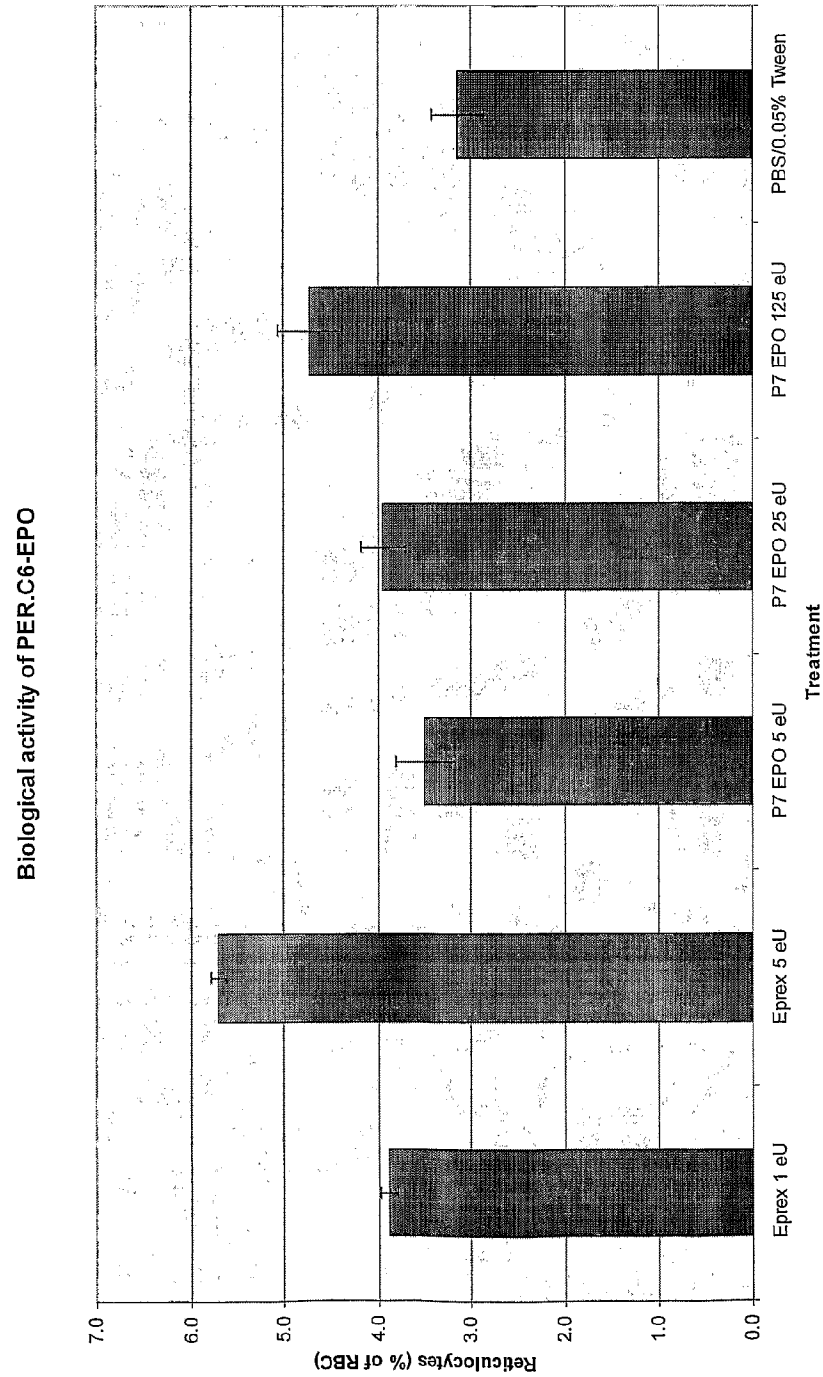
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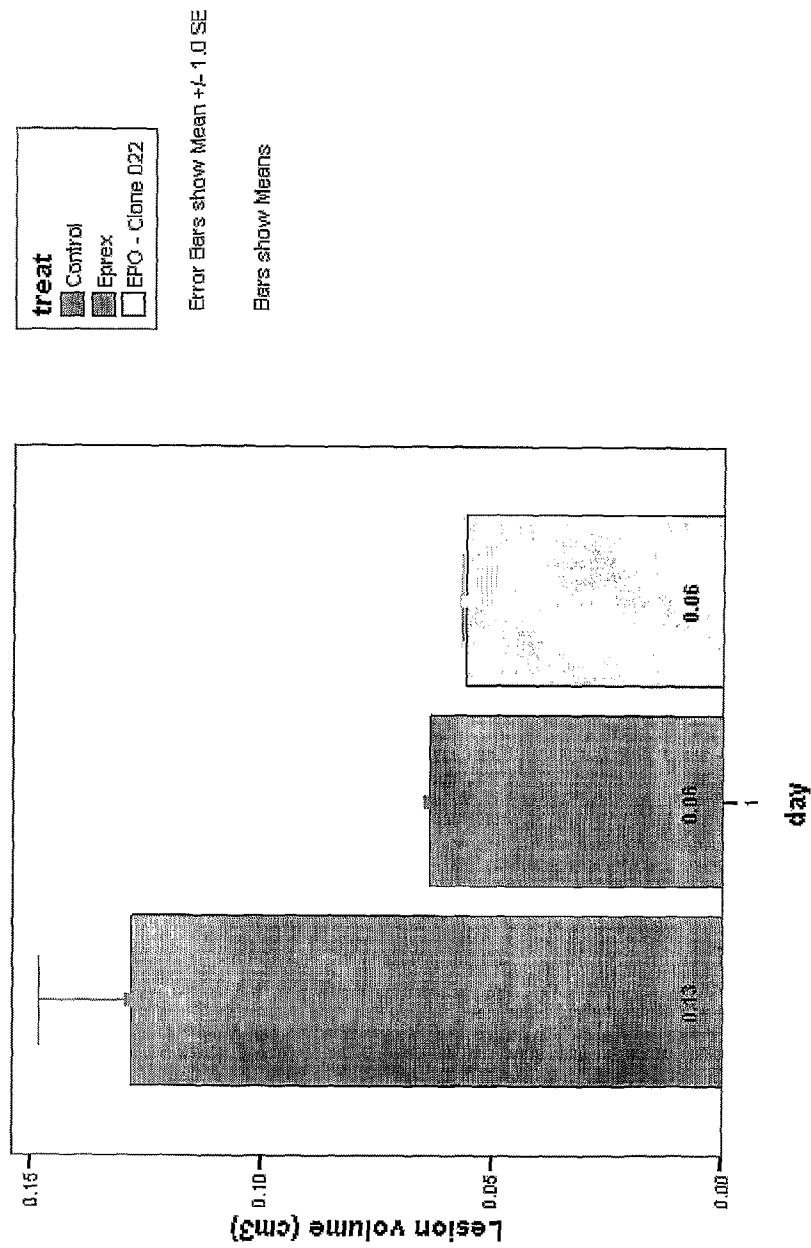
Fig. 7A

T2 maps 24h after stroke
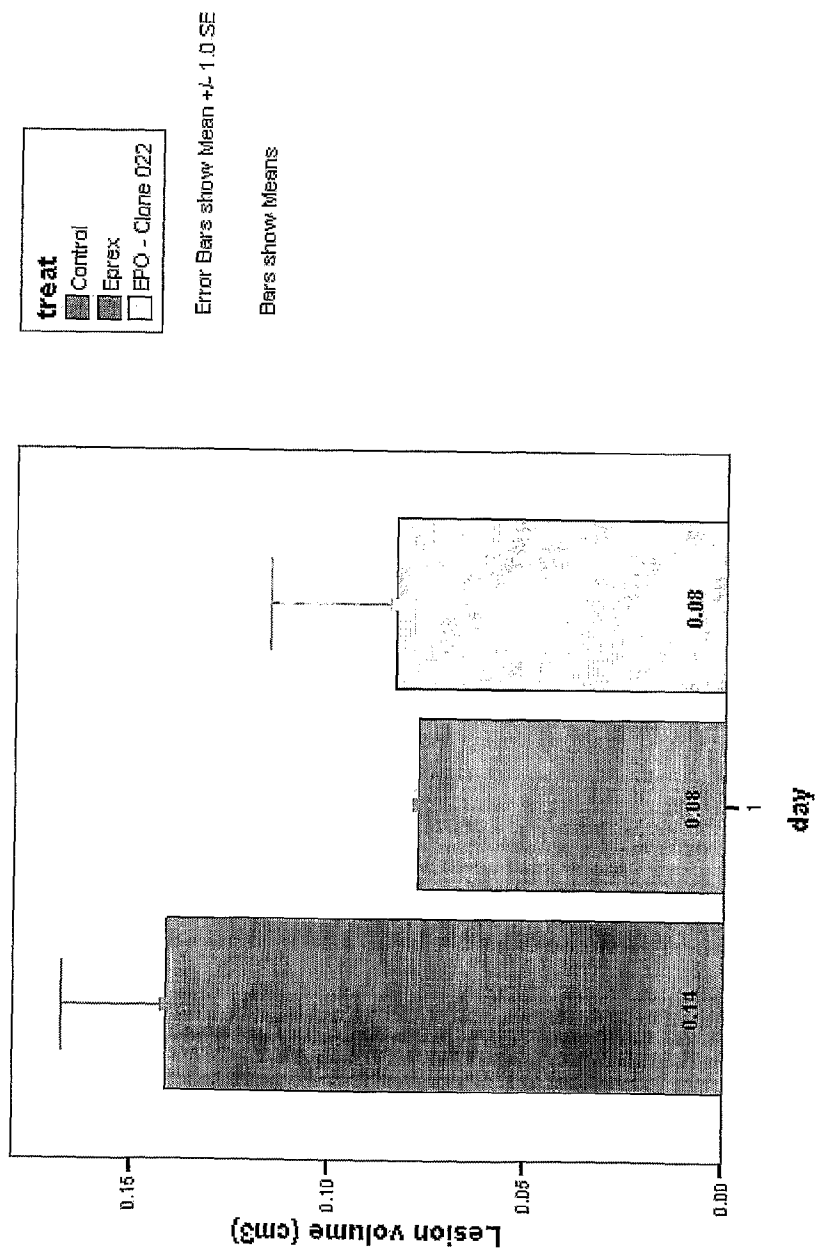
Fig. 7B
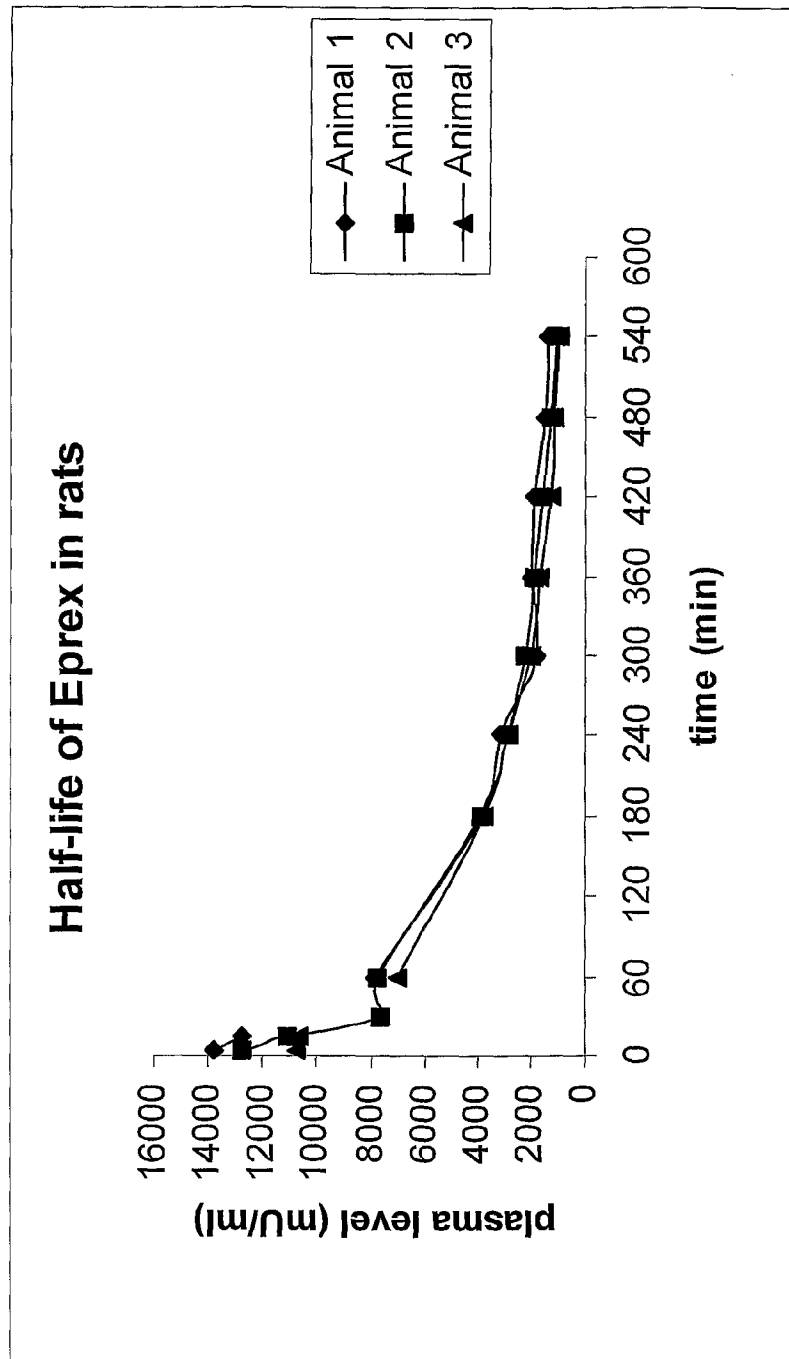
Fig. 8

# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7    C07K14/505    A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7    C07K    A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, PAJ, WPI Data, MEDLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 00 63403 A (SCHOUTEN GOVERT JOHAN ;HATEBOER GUUS (NL); BOUT ABRAHAM (NL); INTR) 26 October 2000 (2000-10-26) | 1-65 |
| Y | the whole document | 66-112, 119-135 |
| X | US 5 856 298 A (STRICKLAND THOMAS WAYNE) 5 January 1999 (1999-01-05) | 1-33, 35-50, 52-61 |
| Y | the whole document | 66-112, 119-135 |
| Y | WO 00 61164 A (KENNETH S WARREN LAB) 19 October 2000 (2000-10-19) the whole document | 66-112, 119-135 |

☐ Further documents are listed in the continuation of box C.          ☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 November 2002 | 03/12/2002 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016 | Wimmer, G |

Form PCT/ISA/210 (second sheet) (July 1992)

**FURTHER INFORMATION CONTINUED FROM     PCT/ISA/ 210**

Continuation of Box I.1

Although claims 105-120 are directed to a method of treatment of the
human/animal body, the search has been carried out and based on the
alleged effects of the composition.

_____

Continuation of Box I.1


Rule 39.1(iv)  PCT - Method for treatment of the human or animal body by
therapy

## INTERNATIONAL SEARCH REPORT

| Box I | Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet) |
|---|---|

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:     –
   because they relate to subject matter not required to be searched by this Authority, namely:

   see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II | Observations where unity of invention is lacking (Continuation of item 2 of first sheet) |
|---|---|

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**     [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

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